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# Characterizing and Restoring Urea Cycle Function in Human Hepatoblastoma-Derived Cell Lines For Use in Bioartificial Liver Devices

Thesis submitted for the degree of Doctor of Philosophy  
(PhD)

Demetra Mavri-Damelin

2007

Centre for Hepatology  
Royal Free and University College Medical School  
London

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## Abstract

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The creation of a bio-artificial liver device could assist liver failure patients awaiting liver transplantation or regeneration by temporarily replacing liver function. The bio-artificial liver device being designed in our laboratory utilises the hepatoblastoma-derived HepG2 cell line. Despite limited liver specific functions whilst in monolayer culture, cell performance can be significantly improved by alginate encapsulation. However, urea cycle activity, which is the mechanism of ammonia detoxification, remains minimal and thus poses a problem in the removal of nitrogenous waste.

The aims of this study were to i) investigate the causes for urea cycle dysfunction in the HepG2 and HepG2-C3A cell lines, and ii) investigate the possibilities of restoring urea cycle function.

To accomplish these, urea cycle gene expression was determined by real time RT-PCR. Protein expression and functional enzyme activity were assessed with Western blotting, metabolic labelling and enzyme assays. To restore function, gene transfer techniques were employed using pcDNA3.1 vectors. In addition, demethylation studies and the adaptation of HepG2 cells to different growth media were carried out.

The main findings of this study showed that the lack of urea cycle function in both HepG2 and HepG2-C3A cell lines is attributable to the under-expression of two urea cycle genes, namely ornithine transcarbamylase and arginase I, although three other enzymes carbamoyl phosphate synthase I, argininosuccinate synthetase and argininosuccinate lyase are present, albeit at lower levels than in primary human hepatocytes. HepG2 cells transfected with plasmid vector constructs containing ornithine transcarbamylase and arginase I full length cDNA, led to the restoration of the urea cycle.

In conclusion, this strategy could be used to restore urea cycle function in HepG2 cells by creating stable cell lines. Future work could aim at determining whether expression of the two constructs is retained over time, and whether this approach provides suitable ammonia detoxification required of a bioartificial liver device.

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## List of Abbreviations

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3D	3-dimensional
50:50 medium	50% complete medium and 50% conditioned medium from HepG2 cells
$\alpha$ -MEM	alpha Minimal Essential Medium
ADC	Arginine Decarboxylase
Arg I	Arginase I
ASS	Argininosuccinate Synthetase
ASL	Argininosuccinate Lyase
ATP	Adenine Triphosphate
BAL	Bioartificial Liver
CaCl <sub>2</sub>	Calcium Chloride
CPM	Counts Per Minute
CPS	Carbamoyl Phosphate Synthase
DEPC	Diethylpyrocarbonate
DMEM	Dulbeccos Minimal Essential Medium
DMF DMA	Dimethylformamide Dimethylacetal
DMSO	Dimethyl Sulfoxide
DPM	Disintegrations Per Minute
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol tetraacetic acid
FCS	Foetal Calf Serum
GC-MS	Gas Chromatography-Mass Spectrometry
GF	Growth Factors
GS	Glutamine Synthetase
HBSS	Hanks Balanced Salt Solution
HCl	Hydrochloric Acid
HEPES	4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid
HG	High Glucose $\alpha$ -MEM medium
HRP	Horseradish Peroxidase
Hyg	Hygromycin
KCl	Potassium Chloride
KCN	Potassium Cyanide

KH <sub>2</sub> PO <sub>4</sub>	Potassium Phosphate
KHB buffer	Krebs Henseleit Buffer
LB	Luria Bertani
LSS	Liver Support System
MCS	Multiple Cloning Site
MOPS	3-(N-Morpholino)propanesulfonic acid
MgCl <sub>2</sub> .6H <sub>2</sub> O	Magnesium Chloride 6 hydrate
NaCl	Sodium Chloride
NaCN	Sodium Cyanide
NADH	Nicotinamide Adenine Dinucleotide
NAG	N-acetyl glutamate
NAGS	N-acetyl Glutamate Synthase
NaHCO <sub>3</sub>	Sodium Bicarbonate
NH <sub>4</sub> Cl	Ammonium Chloride
NO	Nitric Oxide
nor-NOHA	N <sup>ω</sup> -Hydroxy-nor-L-Arginine
NOS	Nitric Oxide Synthase
nsp	Non-specific product
NTC	No template control
ODC	Ornithine Decarboxylase
OPD	o-Phenylenediamine
OTC	Ornithine Transcarbamylase
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulphonyl fluoride
RCCS	Rotary Cell Culture System
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDH	Succinate Dehydrogenase
SDS	Sodium Dodecyl Sulphate
SOC	Super Optimal broth for Catabolite repression
TLC	Thin Layer Chromatography
UV	Ultra Violet
Wnt	Wingless-Int
Zeo	Zeocin

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## Synopsis of Thesis

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### Chapter 1: Introduction

An overview of the structure and functions of the liver, focusing on nitrogen metabolism and the urea cycle.

### Chapter 2: General Methods

### Chapter 3: Characterizing Urea Cycle Function in HepG2 and C3A Cells

To first determine whether urea could be produced by HepG2 and C3A cells, a standard biochemical urea nitrogen assay was used; however, this technique was too insensitive. Gas Chromatography-Mass Spectrometry (GC-MS) was then used, and found that urea was produced by C3A cells but not HepG2 cells.  $^{14}\text{C}$ - $\text{NaHCO}_3$  labelling looked at the incorporation of the  $^{14}\text{C}$  label into urea and urea cycle intermediates. This showed that there was minimal incorporation by both HepG2 and C3A cells, as determined by Thin Layer Chromatography (TLC). As these two experiments were in contrast, cells were incubated with  $^{15}\text{NH}_4\text{Cl}$  to look specifically at ammonia detoxification. GC-MS analysis showed that there was no incorporation of the labelled substrate into urea, which conclusively proved that ammonia could not be incorporated into urea via the urea cycle, in either cell line.

Gene expression, by Real Time RT-PCR, was then carried out on HepG2 and C3A cells to show that whilst three urea cycle genes carbamoyl phosphate synthase I (CPSI), argininosuccinate synthase (AS) and argininosuccinate lyase (AL) were expressed, albeit at lower levels than those in human hepatocytes or human liver, there was a complete absence of ornithine transcarbamylase (OTC) and arginase I mRNA.

Enzyme assays were subsequently carried out to determine the functionality of the urea cycle enzymes. These findings, similar for both HepG2 and C3A cells, supported the mRNA data whereby the functional presence of CPSI, AS and AL, and the absence of OTC were confirmed, although arginase activity was detected in cell lysates, which is contrary to the mRNA data. Since the arginase assay involved detecting the catabolism of  $^{14}\text{C}$ -arginine to  $^{14}\text{C}$ -urea, it was hypothesized that other arginine metabolising enzymes could be responsible for the production of  $^{14}\text{C}$ -urea. Of particular interest was arginase II, an isoform of arginase I. Whilst not found in normal human liver, it is

expressed in other organs, where it converts arginine to urea.

#### **Chapter 4: Alternative Mechanisms of Urea Production**

In this chapter, possible alternative mechanisms of urea production that could account for the arginase activity and urea detected in HepG2 and C3A cells were investigated.

Firstly, arginase II was investigated to determine whether it was responsible for the arginase activity seen during enzyme assays in Chapter 3. This enzyme is involved in metabolising arginine for polyamine and glutamate synthesis with urea produced as a byproduct, and is not part of the urea cycle. Since arginase II is mitochondrial, whereas arginase I is cytoplasmic, the arginase assay was repeated on the two cell fractions. This showed that for both HepG2 and C3A cells, arginase activity was evident only in the mitochondrial fraction. Real time RT-PCR showed raised arginase II expression in both cell lines. Furthermore, Western blotting showed definitively that no arginase I protein is expressed by either of these cell lines; in contrast, arginase II is highly expressed. In addition, mRNA expression levels of agmatinase, which also produce urea via arginine metabolism, were slightly elevated in comparison to primary human hepatocytes and human liver.

To determine the significance of the contribution to urea production by arginase II, inhibition studies were carried out. Arginase activity was inhibited using nor-NOHA and the conversion of  $^{15}\text{N}$ -arginine to  $^{15}\text{N}$ -urea determined. This showed that almost all urea detected from arginine metabolism is from arginase II activity.

#### **Chapter 5: Restoring Urea Cycle Function**

Demethylation, adaptation to arginine deficient medium and gene transfer of OTC and arginase I were explored in an attempt to restore some urea cycle function to HepG2 cells, thereby maintaining ammonia detoxification via this route

Cells were incubated with the demethylating agent 5-azacytidine resulted in the re-expression of OTC mRNA but not arginase I. An alternative argument for the suppression of arginase I could be the presence of increased HNF-4, a hepatocyte transcription factor, in HepG2 cells, and this was confirmed by Real Time RT-PCR.

HepG2 cells were adapted to arginine deficient medium, which has up-regulated OTC

expression in rat hepatoma cell lines. HepG2 cells, however, could not be successfully adapted to this medium.

A gene transfer approach was then investigated to determine transfection of OTC and arginase I could restore some urea cycle function in the HepG2 cell line. OTC and arginase I cDNA was amplified from human liver, ligated into pcDNA3.1 vectors and co-transfected into HepG2 cells. Transient transfections showed that  $^{14}\text{C}$ -urea and  $^{14}\text{C}$ -labelled urea cycle intermediates could be detected by TLC. This seemed a viable method of restoring some urea cycle function and therefore stable cell lines were created. Real Time RT-PCR showed the presence of increased OTC and arginase I expression. Cells in monolayer and 3D culture incubated with  $^{15}\text{NH}_4\text{Cl}$  could produce  $^{15}\text{N}$ -urea, seen using GC-MS.

## **Chapter 6: General Discussion**

An overview of the data presented in each chapter is presented and discussed. Future considerations and experiments are also explored.

## **Chapter 1: Introduction**

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The following study investigates ammonia detoxification and urea cycle function in the human hepatoma-derived cell lines HepG2 and C3A. The work is relevant to the development of bioartificial liver devices, where provision of ammonia detoxification is currently insufficient. This chapter gives an overview to i) the liver, its structure, function and diseases, ii) artificial liver support devices, and iii) nitrogen metabolism, ammonia detoxification and the urea cycle.

### **1.1 The Liver**

The liver is a major site for a vast number of processes that are required to maintain metabolic homeostasis. The functions of the liver are varied and to achieve this, the liver is comprised of different classes of cells, parenchymal and non-parenchymal. These cells carry out functions based upon their location in the liver and also interact with each other. The following section gives a summary of the cell types that comprise the liver, their functions, and briefly, liver structure.

#### **1.1.1 Structure of the Liver**

The human liver, one of the largest organs in the body, is located on the right hand side of the upper abdomen. The liver has a dual blood supply; one arterial and the other venous. The hepatic artery which branches off the aorta, carries in oxygenated blood. The venous blood supply comes through the hepatic portal system and carries nutrient rich blood; having initially passed the small and large intestines, the pancreas and the spleen. This blood from the digestive tract requires immediate processing since it is high in toxic material from the digestive process but also rich in nutrients. The blood leaves the liver via the hepatic vein, and is routed to the heart in order to be pumped through the lungs to be oxygenated (Figure 1.1, page 20). Most waste products from the liver are removed in the hepatic bile duct that joins with the duct coming from the gallbladder; these form the common bile duct, which empties into the duodenum. Other waste is transported via the blood to the kidneys, where it is then excreted in the urine (1).

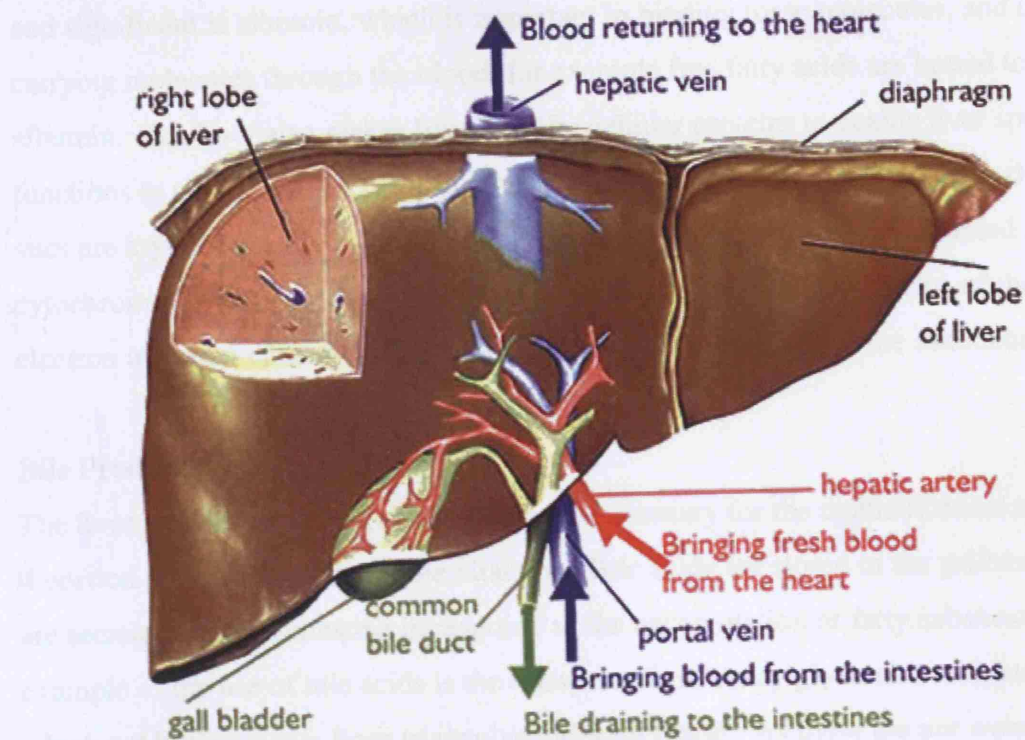


Figure 1.1: The liver. A diagram of the liver showing the flow of blood, oxygenated (red) and deoxygenated (blue), and bile (green), ([www.liverdoctor.com/02\\_liverdetox.asp](http://www.liverdoctor.com/02_liverdetox.asp)).

Within the liver, a network of capillaries is required to both feed and remove products from the cells of the liver. These together form the smallest singular structure in the liver, the acinus (2). The significance of the arrangement of cells in the acinus is discussed further in section 1.1.5 Zonation.

### 1.1.2 Parenchymal Cells

Hepatocytes are the main constituent, ~70%, of the liver mass. These cells are responsible for the majority of functions carried out by the liver and these can be grouped into one of three main categories, i) synthesis, ii) metabolism and iii) detoxification. Outlined below are some important functions that fall within these categories.

#### 1.1.2.1 Synthesis

##### Protein Synthesis

Many proteins, including alpha-1-antitrypsin, fibrinogen, prothrombin and other blood clotting factors, are synthesized and secreted by the liver. One of the most abundant

and significant is albumin, which is important in binding toxic molecules, and in carrying molecules through the blood, for example free fatty acids are bound to albumin. The liver also makes liver specific cellular proteins to enable liver specific functions to take place. Heme is synthesized in virtually all tissues, although the main sites are erythroid cells and hepatocytes. In hepatocytes, heme is incorporated into the cytochromes which are responsible for detoxification, and in components of the electron transport chain in energy maintenance systems such as in the mitochondria.

### **Bile Production**

The liver manufactures bile acids, which are necessary for the emulsification and digestion of lipids and fat soluble vitamins. Bile acids are stored in the gallbladder and are secreted into the intestine in response to the accumulation of fatty substances. One example of the use of bile acids is the transport of monoacylglycerides and fatty acids, which are broken down from triacylglycerides by lipase. As these are not water soluble compounds, they are removed by conjugation with bile acids to form mixed micelles. This facilitates the diffusion of the lipid breakdown products into intestinal epithelial cells. The liver can also remove fat soluble toxins from the body by dissolving them in bile acids, which can then be more easily excreted (3).

### **1.1.2.2 Metabolism**

#### **Glucose Metabolism**

Blood glucose levels need to be kept constant throughout times of feeding and fasting. When there is an increase in blood glucose levels, insulin (produced by the pancreas) causes the excess glucose, coming in from the gut, to be turned into glycogen and stored in the liver. Muscle and fat also take up glucose, where it is converted and stored as glycogen and triglycerides respectively. Insulin also acts to suppress hepatic gluconeogenesis (the formation of glucose) and glycogenolysis (the breakdown of glycogen to glucose). Conversely, when blood glucose levels need to be raised, glucagon (produced by the pancreas) stimulates gluconeogenesis and glycogenolysis in order to release glucose into the blood. At times of starvation when glycogen stores are exhausted, fatty acids are released from adipose tissue to be used for energy. Eventually, muscle protein is broken down to release amino acids, which the liver can use to make glucose (3).

## **Lipid Metabolism**

The liver also synthesises cholesterol, which, along with triacylglycerol, is transported to other organs in the form of very low density lipoproteins, intermediate density lipoproteins, low density lipoproteins and high density lipoproteins. Each lipoprotein is associated with its own set of apolipoproteins, which facilitate the uptake of the lipoprotein by the target cell. They are also required to activate enzymes involved in lipoprotein metabolism. Cholesterol, for example, is essential for cell membranes and is the precursor to bile salts and steroid hormone synthesis (3).

Another essential role of the liver, during times of starvation, is to generate energy by the conversion of some of the fatty acids released from fat into ketone bodies. Other tissues, in particular the brain and heart, can adapt to using ketone bodies for energy. The liver is also a major site of fatty acid synthesis, which is assembled into triacylglycerol and stored mainly in adipocytes (3).

## **Amino Acid Metabolism**

Protein from the diet is broken down into amino acids and many of these are re-synthesized into proteins. However, excess amino acids cannot be stored, therefore what is not absorbed must be metabolised. When amino acids are metabolised, ammonia is released, which is highly toxic and cannot be transported freely through the body. To overcome this, the ammonia is incorporated into glutamine, glutamate or alanine by transamination so that it can be transported to the liver, where the ammonia is then released from these amino acids, and converted into urea to be eventually excreted via the kidneys into urine. The remainder of the metabolized amino acid molecule is used in gluconeogenesis (3).

### **1.1.2.3 Detoxification**

#### **Detoxifying Exogenous and Endogenous Compounds**

Detoxification of chemical substances is carried out by the hepatocytes. This process requires in many cases in two phases. Phase I involves the cytochrome P450 superfamily of enzymes found primarily in the liver, with different isoforms expressed in various zones (4). These microsomal membrane bound haem proteins act on various exogenous (e.g. xenobiotics, alcohol) and endogenous (e.g. steroids, bile acids) compounds in order to detoxify and eliminate them from the circulation. These



enzymes catalyse mono-oxygenation reactions, which form groups that then allow for the conjugation of water soluble compounds by phase II enzymes. In some cases, this primary modification can result in the formation of products even more toxic than the parent compound. Phase II enzymes, in the cytosol, then conjugate the compound by glucuronidation, sulphation, and, conjugation to glutathione and other amino acids. This results in decreasing the reactivity and increasing the water-solubility of the compound so that they can be excreted in the bile or in urine.

## **Modification**

The liver also modifies compounds such as hormones, with one such example being that of the modification thyroxine (T4) to tri-iodothyronine (T3). The liver converts the thyroid hormone T4 into its more active form T3. Thyroid hormones are responsible for altering basal metabolic rate and hence the rate at which biochemical reactions occur in the body, metabolism of cholesterol and bile acids, and growth, amongst many other functions. If T4 is inadequately converted to T3 by the liver, this may lead to hypothyroidism (5).

### **1.1.3 Non-Parenchymal Cells**

There are three main types of non-parenchymal cells in the liver. These are Kupffer cells, Stellate cells, and Sinusoidal Endothelial cells. Kupffer cells are the macrophages of the liver and are derived from monocytes. In the liver they produce cytokines and inflammatory mediators, and are involved in cytotoxic destruction of organisms and some tumour cells. They are also believed to be the main source of TNF $\alpha$  production which is an important mediator of liver injury (6;7). They may also be involved in liver regeneration (8). Stellate cells produce extracellular matrix, synthesize growth factors, and produce cytokines. They are also the storage site for vitamin A (9;10). Sinusoidal Endothelial cells have been shown to produce lymphocyte co-stimulatory molecules and allow leukocytes to enter the liver. They also form tissue lining in the form of fenestrations that act like a sieve barrier, which allows the sinusoidal endothelium to regulate the passage of particles less than 10nm in diameter from the blood to the underlying cells in the liver parenchyma. Therefore, solutes in the blood can be exchanged with those in the hepatocytes (11).

### 1.1.4 Hepatic Stem Cells

Theoretically activated upon insult or injury to the liver, hepatic stem cells are thought to be one reason why the liver can recover after injury and regenerate. Many groups have tried to isolate hepatic stem cells and characterize them, however this has proved very challenging as not only are they difficult to isolate, but as there is not a defined set of markers that characterize a stem cell, they can only suggest a particular phenotype (12;13). In addition, the existence of liver stem cells was brought into question by studies which showed that bone marrow stem cells can fuse with hepatocytes to form a fusion cell that displays characteristics of both cell types and carries out the functions associated with stem cells (14).

### 1.1.5 Zonation

Zonation describes the existence of varying 'zones' in the liver, which are classed as: zone 1= periportal; cells located around the portal vein and arterioles, zone 3= perivenous; located around the central vein and terminal veins, and zone 2; cells which lies in between zones 1 and 3 (Figure 1.2). The effects of zonation are seen since hepatocytes and non-parenchymal cells located in different areas of the acinus are exposed to differing concentrations of oxygen, metabolites, hormones and toxins (15;16). This is dependent upon where cells lie in relation to vessels that provide nutrient and oxygen rich blood, and vessels responsible for removing blood and waste products (17).

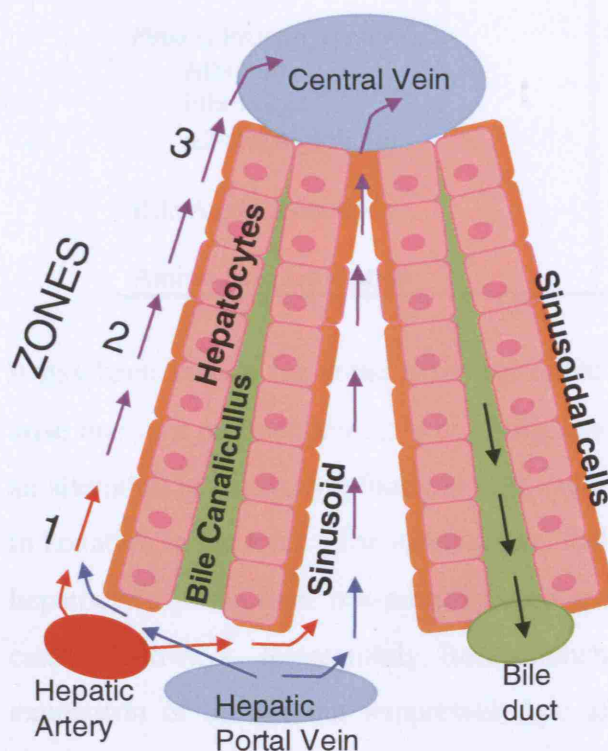


Figure 1.2: The liver acinus.

The zones one to three depend on the distance of the hepatocytes from the hepatic artery and portal vein, and their proximity to the central vein. Nutrient-rich blood from the portal vein (blue arrows) mixes (purple arrows) with oxygen rich blood from the hepatic artery (red arrows), this then drains into the central vein.

Hepatocytes in the periportal area are exposed to oxygen and nutrient rich blood and hepatocytes in the perivenous region are exposed to CO<sub>2</sub> and metabolite rich blood. To cope with this, hepatocytes in the two zones have differing compositions of enzymes and organelles, for example, periportal hepatocytes contain larger but fewer mitochondria than perivenous hepatocytes (18). The various functions carried out by hepatocytes located in the two regions are summarised in Table 1.1. Non-parenchymal cells are also subject to zonation (Table 1.2, page 26).

*Table 1.1: Zonation of parenchymal cells. Examples of functions carried out by parenchymal cells in the periportal and perivenous zones in the liver.*

<b><i>Periportal</i></b>	<b><i>Perivenous</i></b>
Oxidative Energy Metabolism Fatty Acid Oxidation Citrate Cycle	
Cholesterol Synthesis	Ketogenesis
Gluconeogenesis Glycogenolysis	Glycolysis Lipogenesis Glucose to glycogen
Glutathione Peroxidation	Xenobiotic Metabolism by Mono-oxygenation (Cytochrome P450s) and Glucuronidation
Ureagenesis	Glutamine formation by Glutamine synthetase
Plasma Protein Synthesis: Albumin Fibrinogen $\alpha$ 2-macroglobulin	Plasma Protein Synthesis: $\alpha$ -1-antitrypsin $\alpha$ -fetoprotein Angiotensinogen
Bile Acid Production	
Amino Acid Utilization	

It has been known for some time that some forms of hepatocellular carcinoma may arise due to a malfunctioning Wnt (Wingless-Int)/ $\beta$ -catenin pathway, accompanied by an alteration in hepatocyte function (19) and recently, this pathway has been implicated in zonation in the liver. The mechanisms that may control this heterologous pattern of hepatocytes in the liver has recently been attributed to a balance between the Wnt/ $\beta$ -catenin pathways. Interestingly, Benhamouche *et al.* have shown that disruption in the expression of the tumour suppressor Apc affects Wnt/b-catenin signalling and thus

alters the periportal/pericentral phenotype (20).

*Table 1.2: Zonation of non-parenchymal cells.*

<b><i>Cell Type</i></b>	<b><i>Periportal</i></b>	<b><i>Perivenous</i></b>
Kupffer	Greater cell number Lysosomal enzymes secreted Phagocytosis	Cytotoxic capacity
Stellate	Greater cell number Matrix formation Lipid droplets: Vitamin A storage Growth factors	
Endothelial	Endocytosis via lectin binding	Filtering due to an increase in fenestrae number

## **1.2 Disease and Treatment**

### **1.2.1 Toxin Induced Injury**

The liver is exposed to a myriad of chemicals that it has the ability to detoxify or recover from. However, very high doses of a hepatotoxin such as paracetamol overdose can quickly lead to liver failure, or acute liver disease. Long term abuse of a toxic substance such as alcohol can lead to chronic liver disease. High alcohol intake can also lead to fatty liver, where excess fat accumulates in fat cells causing an enlarged liver; alcoholic hepatitis, where the liver is acutely inflamed and liver cells are destroyed; and alcoholic cirrhosis where normal liver tissue is replaced by scar tissue that does not have hepatocyte function (1).

### **1.2.2 Viral Induced Injury**

The hepatitis virus, of which there are five main types Hepatitis A, B, C, D and E, infect the liver specifically and can cause inflammation and scarring of the liver. Patients can be treated with drugs but persistent infection can lead to chronic hepatitis whereby the liver sustains inflammation and damage (1).

### **1.2.3 Liver Tumours**

Liver tumours can be both benign, as in the form of hepatocellular adenoma or hemangioma, or can be malignant. Hepatomas, or hepatocellular carcinoma, can be

caused by chronic viral infection, chronic exposure to hazardous chemicals such as carbon tetrachloride and alcohol abuse. Other liver cancers that are less common include cholangiocarcinoma, hepatoblastoma and angiosarcoma. Many tumours are operable, providing that they are confined to a particular region of the liver and are not metastatic. They can then be removed by surgery and the patient treated with chemotherapy or radiotherapy. The liver can eventually regenerate back to its normal size, accompanied by a return to normal function (1).

#### **1.2.4 Metabolic Disorders of the Liver**

Two examples of metabolic disorders that affect the liver result from the accumulation of metallic ions are hemochromatosis and Wilson's disease, which cause eventual liver failure and organ damage. Hemochromatosis results from excess iron absorption and storage mainly in the liver and pancreas. Wilson's disease results from the accumulation of copper in the liver. Other common metabolic disorders of the liver include the hypercholesterolaemias, where the liver cannot adequately remove cholesterol; glycogen storage diseases, which affect the storage and metabolism of glycogen; and defects in bile production. The most common inherited metabolic diseases are caused by urea cycle defects. As these affect ammonia detoxification, patients rapidly develop hyperammonaemia and hepatic encephalopathy. Whilst they occur with varying degrees of severity and can be managed by diet and drugs, the most serious cases can be fatal within days of birth. Ornithine transcarbamylase (OTC) deficiency is the only X-linked urea cycle defect and most serious (21).

#### **1.2.5 Treatment**

Many of the above diseases are treated and managed with the relevant drugs. However, if neglected, the liver often becomes fibrotic and cirrhotic. Normal liver tissue is replaced by fibroblasts, which leads to liver function being progressively lost and blood flow throughout the liver being diminished. Eventually the only treatment available for some of these chronic diseases is liver transplantation as the liver is too damaged for regeneration. There are also a vast number of patients that suffer from acute diseases caused by, for example, a short term toxic assault on the liver. In these cases, liver failure and loss of liver function results from damage to liver cells and not from fibrosis or cirrhosis (1). Over the years, many people have worked on trying to develop artificial liver support that could replace some of the functions of the liver whilst these patients await transplantation or liver regeneration.

### 1.3 Artificial Liver Support

Artificial liver support systems offer the prospect of short term assistance and support to liver function in patients with liver failure. They are designed to serve as a bridge to liver transplantation, or liver regeneration. There are two types of such devices currently in development, i) cell-free devices and ii) bioartificial liver devices.

#### 1.3.1 Cell-Free Devices

Cell free liver support devices work on the principle that toxins that cause hepatic encephalopathy and organ failure can be removed from the plasma, as opposed to using cells to actively metabolize them. This is currently done using one, or a combination of, the following methods: i) filtering large molecules using a membrane, ii) exchange diffusion of small molecules across a membrane (dialysis), and iii) adsorbing toxins using charcoal, resins, or albumin. Examples of such devices are summarised in Table 1.3. Devices that rely on the above mechanisms have had mixed results in clinical trials. One reason for their failure is that whilst they can remove toxic components, they frequently do so non-specifically and moreover, they do not replace proteins produced by the liver, such as many of the plasma proteins and clotting factors. Their detoxification characteristics are also partly inadequate, and they lack metabolic functions. Therefore a number of cell-based liver support systems are undergoing major investigations.

*Table 1.3: Cell-free liver support devices in development.*

Molecular Adsorbent Recirculating System	Recirculating albumin impregnated membrane and dialysate, using charcoal and anion exchange to partially cleanse the albumin.	Some improvement in biochemical and clinical parameters, increased survival in some groups of patients, but not in acute liver failure.	(22;23)
Artificial Liver Support System	Plasma exchange, charcoal hemoperfusion, plasma bilirubin adsorption, charcoal plasma perfusion, hemofiltration and hemodialysis	Some improvement in biochemical and clinical parameters. No apparent increase in survival.	(24)
Prometheus Fractionated Plasma Separation and Absorption	Separation through an albumin-permeable membrane, adsorption using resin and anion exchanger and hemodialysis.	Some improvement in biochemical and clinical parameters. No survival benefit.	(25-28)

### **1.3.2 Bioartificial Liver Devices**

As the name suggests, Bioartificial Liver devices (BAL) consist of a biological component that would substitute key liver functions such as albumin synthesis and ammonia detoxification. The development of such a device is a multistage process that must take into account a number of questions:

- i) Which would be the best choice of cell type?

The choice of a cell source is paramount to the provision of appropriate and sufficient liver functions by BAL systems. There are a number of BAL devices currently in development and undergoing clinical trials, summarized in Table 1.4 (page 31). The biological component, on which these devices rely need to mimic the function of human hepatocytes. Currently, human hepatocytes isolated from pieces of normal liver are not a viable option as they rapidly lose the ability to maintain liver specific functions when in culture; moreover they do not proliferate in culture. Although one machine has been developed using this approach, the difficulties of production mean it is unlikely to become readily or generally available (29). Porcine primary hepatocytes have been used with some success, but they raise immunogenic and functional considerations with a risk of zoonoses; of particular concern is pig endogenous retrovirus (30;31). Liver cell lines are another possibility as they confer many of the characteristics of liver cells. They have the advantage of constant availability and, due to clonality, the variation in functional ability is minimal (32). One concern however, is the possibility that they could cause tumour formation if cells escape from the system, but this can be addressed by using cell-retaining membranes.

Cell lines however are known to not express a complete repertoire of hepatocyte functions, and many cell lines have lost some of their synthetic and detoxificatory functions and can neither fully detoxify chemicals via cytochrome P450 activity nor detoxify ammonia as effectively as hepatocytes, properties that are essential to BAL systems (33-35). This change in function may be due to the tumour-like properties of these transformed cells, where certain functions necessary for tumour cell growth are up-regulated at the expense of others. The role of methylation in cancers is well documented, and in particular the silencing of gene transcription by promoter hypermethylation could cause the loss of gene function (36). Cell lines may also have lost chromosomal material.



This decrease in hepatocyte properties in cell lines has led to investigations into modified culture conditions to up-regulate function. In the case of C3A cells, seen in Table 1.4, the parent cell line HepG2 was adapted to different culture medium, which had the apparent effect of increasing urea production (37;38). In addition the physical manner in which cells are grown can lead to a change in function. Currently, most devices use hollow fibre systems. However others, such as rotary cell culture system (RCCS), 3-dimensional (3D) culture and fluidized bed bioreactors (FBB) all contribute to increasing cell function. Currently, the devices undergoing clinical trials (Table 1.4), have had mixed success (39). Our group is currently developing a BAL system using 3D cultured HepG2 cells in a fluidized bed bioreactor.

ii) How many cells are required to sufficiently replace liver function?

Various groups have estimated this in either terms of mass, i.e. in grams equivalent to a lobe of liver, from 200-600g, or in terms of cell number, from  $5 \times 10^{10}$ . Ammonia levels need to be reduced from  $>100 \mu\text{mol/L}$  to  $<75 \mu\text{mol/L}$  with  $10\text{-}40 \mu\text{mol/L}$  being the normal range. In terms of urea synthesis, it is estimated that a minimum of  $15 \times 10^9$  hepatocytes can produce at least  $5 \mu\text{g urea/hr/million cells}$  ( $0.083 \mu\text{mol/L}$ ), and cells need to achieve this level of urea production to provide adequate ammonia detoxification (31).

iii) How can so many cells be cultured efficiently and be available immediately?

Large scale devices for culturing cells that also increase hepatocyte specific functions are constantly being developed. Cryopreservation is also important for BAL devices.

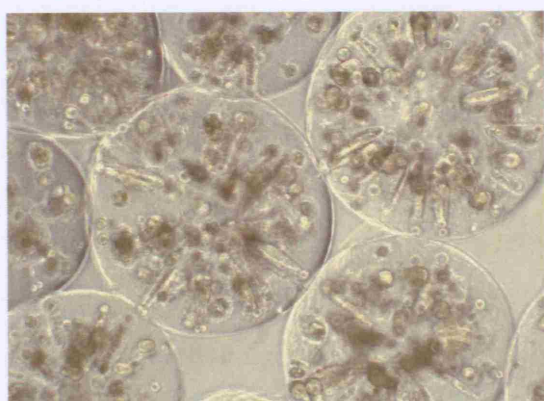
### 1.3.3 Our Device and Choice of Cell Type

Our group has worked on developing a BAL device that will serve as a bridge for patients awaiting liver transplantation or regeneration. The first candidate for the biological component of the BAL was the hepatoma derived cell line LG6. These cells had minimal liver-like function and no urea cycle activity in monolayer. To improve the function of these cells, a 3D culture system was developed whereby cells are encapsulated into alginate beads (40;41). In 3D culture, the function of LG6 cells improved considerably and they also produced urea. However, this cell line was found to contain a rodent retroviral element which was re-expressed as an active retrovirus in the cells grown in 3D culture, making them unsuitable for use in the BAL.

*Table 1.4: Bioartificial Liver Support devices in Phase I clinical trials.*

	Extracorporeal Liver Assist Device	Hepat-Assist	Bioartificial Liver Support System	TECA-type Hybrid Artificial Liver Support System	Radial Flow Bioreactor	Liver Support System	Modular Extracorporeal Liver Support	Hybrid Bioartificial Liver	Amsterdam Medical Centre-Bioartificial Liver
<b>Cell Type</b>	C3A hepatoma-derived	Porcine-cryopreserved	Porcine-freshly isolated	Porcine-freshly isolated	Porcine-freshly isolated	Porcine	Human- freshly isolated	Porcine-freshly Isolated	Porcine-freshly isolated
<b>Cell Mass</b>	200-400g	5-7x10 <sup>9</sup>	7-20g	10-20 x10 <sup>9</sup>	200-230g	Up to 600g	Up to 600g	10x10 <sup>9</sup>	10x10 <sup>9</sup>
<b>Mass Transfer Membrane</b>	70kDa cutoff	0.2µm porous	100kDa cutoff	/	1µm cutoff, polyester	300kDa	400kDa	100kDa cutoff	Direct cell contact
<b>Barrier Filter</b>	1µm	No	No	/	0.4µm	No	No	No	0.4µm
<b>Additional Devices</b>	No	Charcoal Column	No	Charcoal Column	No	No	Albumin Dialyzation	Charcoal or Bilirubin Column	No
<b>Survival Improvement</b>	No	In subgroups	n/a	n/a	n/a	n/a	n/a	n/a	n/a
<b>Neurological Improvement</b>	Probably	Yes	(under sedation)	Yes	Yes	/	Yes	Unclear	(under sedation)
<b>Ammonia Removal</b>	8% increase	18% decrease	33% decrease	/	33% decrease	No change	/	Unclear	44% decrease
<b>Bilirubin Removal</b>	20% increase	18% decrease	6% decrease	/	11% decrease	/	/	Unclear	35%
<b>References</b>	(38,42)	(43-45)	(46-48)	(49;50)	(51)	(52;53)	(29)	(54)	(55-58)

The human hepatoblastoma-derived HepG2 cell line was then considered as a potential source for the cellular component of the BAL. HepG2 cells, like many other liver-derived cell lines, have been used as surrogates for primary human hepatocytes as they express many classic hepatocyte-like characteristics, both synthetic and detoxificatory. In monolayer, HepG2 cells exhibit a limited range of functions, however, some functions not apparent in monolayer cultured cells can be significantly enhanced by encapsulating cells in alginate beads and culturing them in this 3D configuration, where cells form cohesive spheroids (Figure 1.3). An increase in synthetic and some detoxificatory capacity has been shown using microarray and functional studies comparing monolayer to 3D culture (32). Furthermore, when the alginate beads are cultured in an RCCS, HepG2 cell proliferation is further increased thereby increasing the per bead performance. It is thought that less sheer stress and increased mass transfer, due to the microgravity environment induced by this system allows the cells to perform better (41). In addition, alginate encapsulated HepG2 cells can be cryopreserved. Previous studies have shown that these encapsulated cells can indeed improve the symptoms of liver failure (42).



*Figure 1.3: HepG2 cells in alginate. After eight days in culture, HepG2 cells grow in alginate beads to form cohesive spheroids of cells.*

Despite this improvement, limitations to using HepG2 cells remain. Studies have shown that some phase I enzymes are still missing, although phase II enzymes are present. In addition, as with other cell lines, ammonia detoxification is also missing. As many of the enzymes involved in these detoxificatory processes are not up-regulated by 3-D culture, this poses a problem for their use in the development of a BAL. As already discussed, in the case of ammonia detoxification, amino acid catabolism results in the release of ammonia, which the liver rapidly converts via the urea cycle to urea.

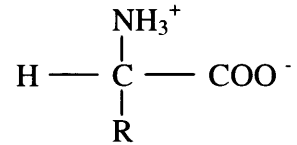
Raised levels of ammonia, which occur rapidly in patients with acute liver failure as they have compromised ammonia detoxification capabilities, can result in a hyperammonaemic state where patients are at the risk of developing hepatic encephalopathy. In addition, glutamine levels in the brain also rise dramatically with detrimental consequences (43-46). Plasma ammonia levels normally lie with the range of 10-47  $\mu\text{mol/L}$ , whereas in patients with acute liver failure, levels can rise to over 170  $\mu\text{mol/L}$  (47). Current devices offer limited ammonia removal as hepatocytes, whether human or porcine lose urea cycle function over time, and whilst the C3A cell line produces urea, BALs using them have not yet been effective. Previous studies have observed that HepG2 cells tend toward removing ammonia via synthesis of glutamine and other groups have attempted to address the lack of ammonia detoxification by HepG2 cells via the urea cycle by enhancing diversion of ammonia into glutamine rather than to urea (48;49). However, there is a concern that this approach could add to the burden of raised ammonia and glutamine in liver failure patients.

One other approach could be the use of drugs that could assist in ammonia removal, such as those used in urea cycle disorders, and in conjunction with reducing ammonia output from gut flora by antibiotic treatment (50). Sodium phenylbutyrate is rapidly metabolized to phenylacetate, which conjugates with glutamine via acetylation to form phenylacetylglutamine (21). This then is excreted by the kidneys and therefore provides an alternative mechanism of nitrogen removal. Sodium benzoate removes ammonia via the conversion of glycine to hippurate (21). There has been some success with sodium benzoate in reducing symptoms of hepatic encephalopathy. However, as the enzymes responsible for these conversions are located in the liver, patients with compromised liver function may not metabolize these drugs appropriately and negative side effects from unregulated levels of drugs in the blood (51;52).

Since the reasons behind the lack of a functional urea cycle in HepG2 cells have not yet been fully elucidated, this study aimed to characterize the extent of urea cycle function in HepG2 cells in order to determine the causes for the down-regulation of activity in this hepatoblastoma cell line. Central to this thesis therefore is the investigation into the lack of urea cycle activity in HepG2 cells and attempts to overcome this problem, discussed further in section 1.6.

## 1.4 Nitrogen Metabolism

Proteins are created from the 20 amino acids that we either absorb from our diet or synthesize. Amino acids have a basic structure, where the R group represents a side chain:



The synthesis and catabolism of these amino acids is unique to each one. This section will cover the basic catabolism of amino acids, which results in the release of the amino group, and the recycling of the carbon chain (3).

Intrahepatic sources of nitrogen are mainly in the form of ammonia due to the deamination of some amino acids. Extrahepatic sources of nitrogen are mainly from the intestine, kidney, and muscle (53). In the intestine, animal studies showed that glutamine nitrogen was converted to ammonia, citrulline and alanine, which were all released into the portal circulation. Citrulline is taken up by the kidney almost at the same rate that it is excreted from the intestine and converted to arginine which is secreted into the circulation. The kidney produces ammonia due to renal glutaminase activity. In the muscle, alanine and glutamine production provide the ammonium releasing precursors for ureagenesis (21). Small amounts of nitrogenous waste can also be excreted in other forms, these are creatinine and urate. The former carries nitrogen from creatine phosphate, ATP and nucleic acid metabolism, and the latter from purine nitrogen derived from ATP (3).

### 1.4.1 Ammonia Removal

When amino acids are catabolized, the ammonia released is toxic, therefore in order to detoxify ammonia rapidly and excrete it, the liver converts ammonia (in the form of ammonium ions since  $\text{NH}_3$  at physiological pH becomes  $\text{NH}_4^+$ ) to urea, which is a non-toxic, highly water-soluble molecule (3). However, ammonia, is not taken up by the liver in its free form but is instead transported to the liver as the amino acids glutamine, alanine and glutamate (Figure 1.4, page 35).

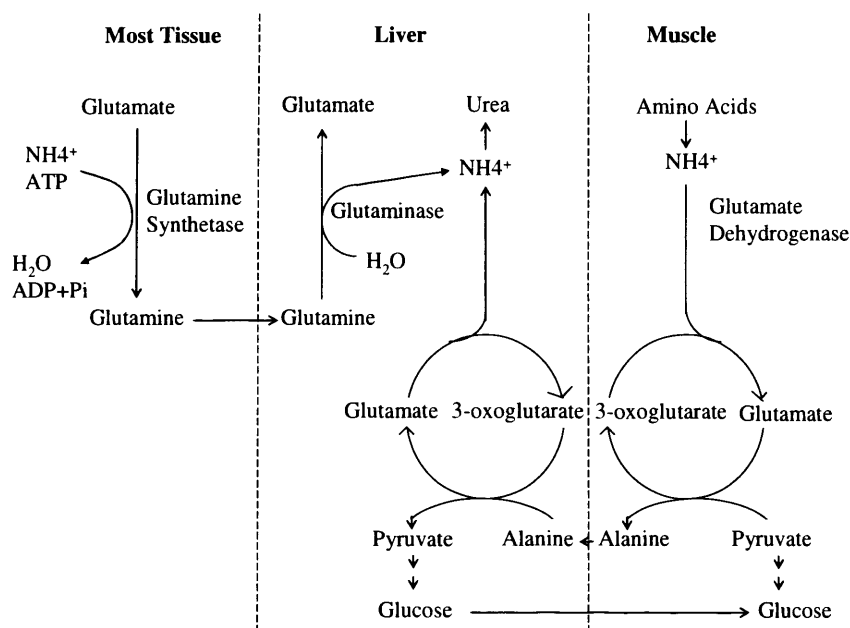


Figure 1.4: Transportation of ammonia through the circulation to the liver to be incorporated into urea.

Ammonia is transferred to glutamate and alanine by transamination (Figure 1.5a step 1). Here,  $\alpha$ -amino groups from amino acids are transferred to 3-oxoglutarate to form glutamate, and in muscle to pyruvate to give alanine. A second free amino group, released during ATP hydrolysis or deamination of glycine, lysine, threonine and serine, forms an amide bond with glutamate to give glutamine (Figure 1.5a, step 2) (3).

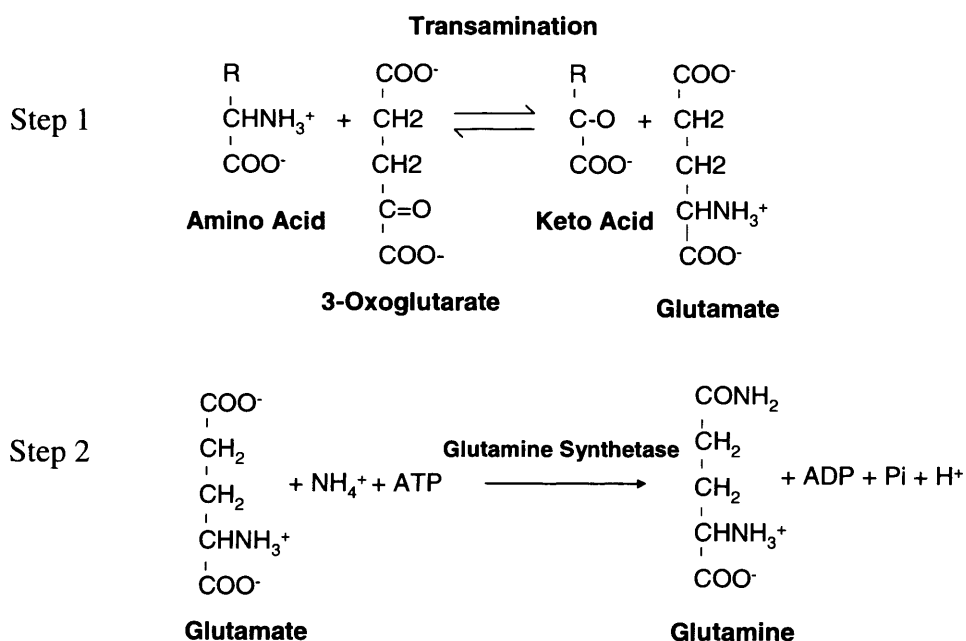
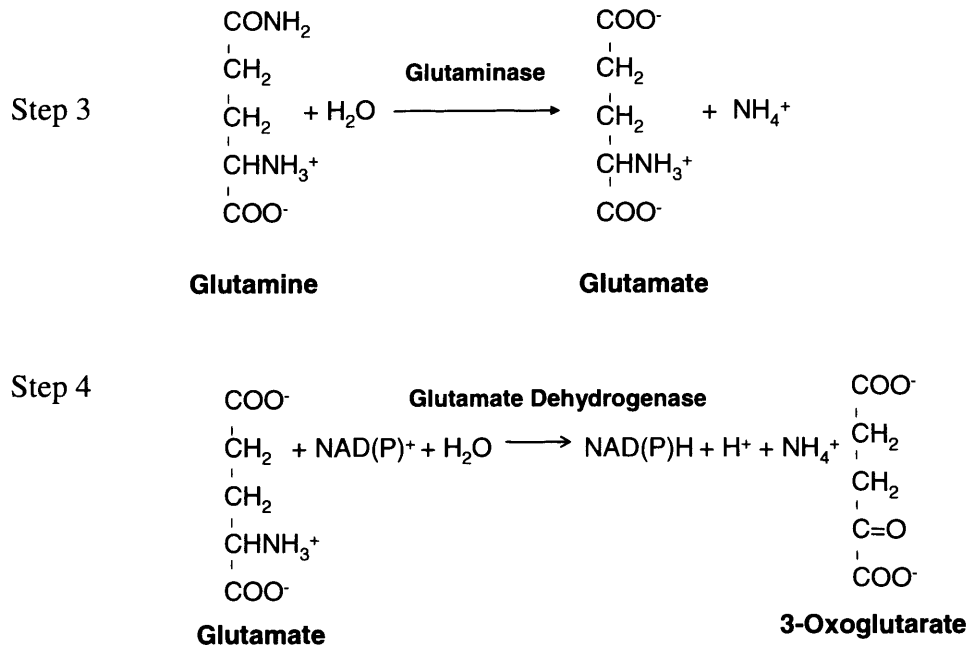


Figure 1.5a: Transamination and glutamine synthesis.



*Figure 1.5b: The production and catabolism of glutamate.*

The ammonium ions, incorporated into glutamine and alanine, can now be released by the peripheral tissue to be taken up by the liver. In the liver, alanine is converted back to glutamate and pyruvate which is used for gluconeogenesis. Glutamine and glutamate are the direct precursors for the release of ammonia into the urea cycle and are transported into the mitochondria of the periportal cells. Glutaminase releases one ammonium ion from glutamine to leave glutamate (Figure 1.5b step 3). Glutamate dehydrogenase releases the ammonium ion from glutamate to leave 3-oxoglutarate (Figure 1.5b, step 4). The ammonium ions now combine with bicarbonate ions to enter the urea cycle (3).

Stable isotope labelling studies have shown that the two nitrogen atoms in urea come from different sources. The first ammonia comes from glutamine and enters the urea cycle from the mitochondria as described above. The second nitrogen for urea is provided by aspartate that can be formed from alanine and glutamate metabolism (Figure 1.6, page 37). Aspartate enters the urea cycle cytoplasmically and is incorporated onto citrulline (54)

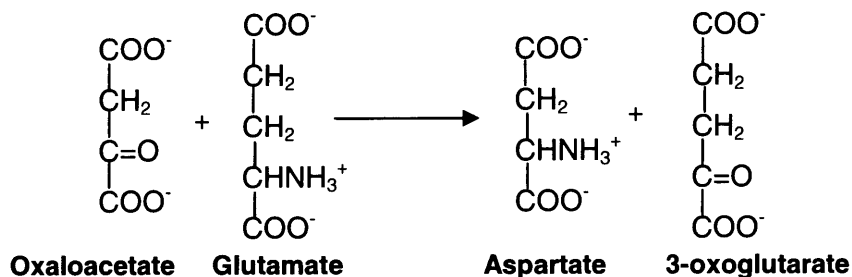


Figure 1.6: Aspartate synthesis.

### 1.4.2 The Urea Cycle

The urea cycle, first identified by Krebs and Henseleit, is a series of successive reactions that serve two main purposes in cellular metabolism. The first is the conversion of toxic nitrogenous compounds to the water-soluble substance urea (55). The second is the *de novo* synthesis and degradation of the amino acid arginine by components of the urea cycle (3). Higher mammals and a number of amphibians detoxify ammonia by converting it to urea. Being less toxic than ammonia, it can be stored in urine in the bladder and excreted. Other animals, such as some fish and amphibian larvae excrete ammonia directly into the surrounding water. Birds and terrestrial reptiles convert ammonia into uric acid that can be excreted in solid form or dissolved in very small amounts of water (56).

The urea cycle consists of a series of enzymes that are responsible for the disposal of waste nitrogen that accumulates as a by-product of amino acid metabolism. The complete cycle only takes place in the liver, although parts of the cycle occur in the endothelial cells of the intestine to produce citrulline, and in the kidney to convert citrulline to arginine. Together, the two organs are responsible for arginine synthesis. Arginine produced by the urea cycle is not released for protein synthesis but is solely for the purposes of removing urea and replenishing the urea cycle with ornithine (3;21).

It has been observed that in the liver, the urea cycle and its associated enzymes are affected by zonation (57). The urea cycle is only carried out by periportal hepatocytes that have a low affinity but high capacity for ammonium ions. Perivenous hepatocytes are rich in glutamine synthetase and act as ammonia scavengers in that they have a high affinity for ammonium ions but low capacity (58). Therefore, if any ammonia escapes the urea cycle from the periportal hepatocytes, the glutamine synthetase (GS) rich hepatocytes that surround the periportal hepatocytes incorporate the ammonia into



glutamine. This is then released back into the circulation to re-enter the periportal region (17;57).

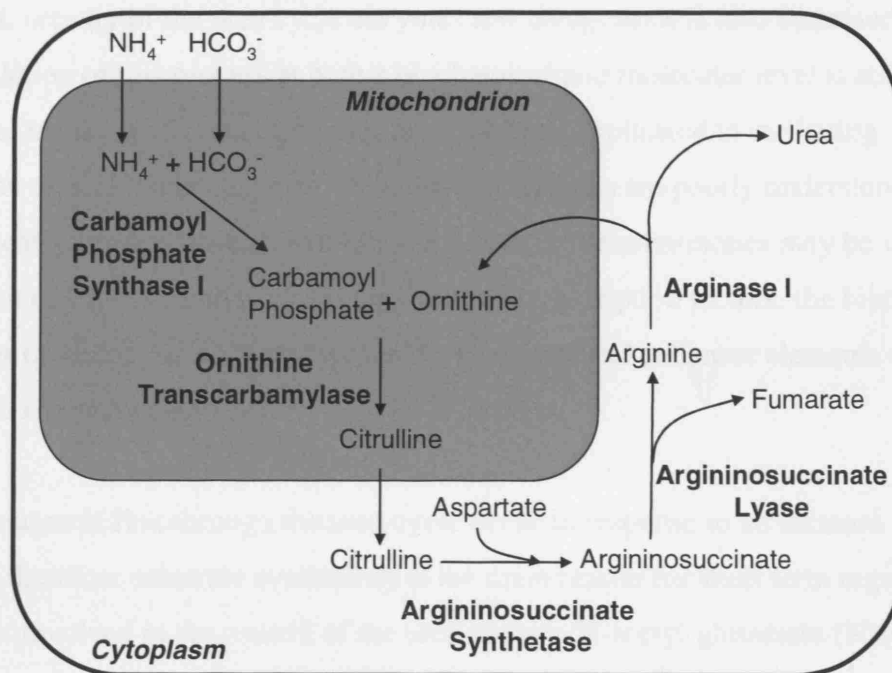


Figure 1.7: The urea cycle.

As seen in Figure 1.7, ammonium ions and bicarbonate ions accumulate in the mitochondria. The first committed step of the urea cycle involves Carbamoyl Phosphate Synthase I (CPSI). This enzyme converts ammonia and  $\text{CO}_2$  to carbamoyl phosphate, which is combined with ornithine, by Ornithine Transcarbamylase (OTC), to give citrulline, which leaves the mitochondria and enters the cytosol. Argininosuccinate Synthetase (AS) adds an aspartate molecule to citrulline to give argininosuccinate. Fumarate is then cleaved from this molecule by Argininosuccinate Lyase (AL) to give arginine. Finally, Arginase I is responsible for the hydrolysis of arginine, releasing ornithine which feeds back into the urea cycle, and urea, which is exported for renal excretion (3).

#### 1.4.2.1 Urea Cycle Regulation

The urea cycle is regulated both by long term and short term modification in order to control changes in nutrition and to prevent the accumulation of toxic metabolites. Long term modification in the cycle involves changes in the levels of enzymes as a consequence of prolonged changes in protein intake. These enzymes are mainly under transcriptional control. Human studies have shown that on a high protein diet (100g

per day), 80% of urea nitrogen comes from dietary sources, on a low protein diet (42g per day), 46% of urea nitrogen comes from this source (21;59). In primates, protein (nitrogen) intake and ureagenesis is directly proportional; when dietary protein is increased, activity of the urea cycle enzymes and ureagenesis is also increased (60). The regulation of this process at both a biochemical and molecular level is still unclear. Glucagon, insulin, and glucocorticoids have all been implicated in mediating expression of urea cycle enzymes, although mechanisms are poorly understood (59;61). More recently, studies have shown that the action of these hormones may be via the interaction of CCAAT/enhancer-binding protein transcription factors, the binding sequences of which can be found within the promoter and enhancer elements of the urea cycle enzyme genes (62).

Rapid changes in flux through the urea cycle occur in response to an increase in waste nitrogen, therefore substrate availability is the main reason for short term regulation. A key factor involved in the control of the urea cycle is N-acetyl glutamate (NAG) which is responsible for activating CPSI and glutaminase. NAG is synthesized from glutamate and acetyl-CoA by N-acetyl glutamate synthase (NAGS), the activity of which increases when the level of amino acids rises. This occurs because of an increase in glutamate saturation and allosteric activation by arginine. During times of fasting, ureagenesis is also increased because of gluconeogenesis from muscle amino acids. In this instance, NAG levels increase by a glucagon signalling mechanism that is not yet fully understood (63-65).

#### **1.4.2.2 Urea Cycle Enzymes**

The urea cycle enzymes detailed below exist as part of a cycle in hepatocytes which converts ammonia into urea (55). However, some also exist in other tissues and cell types, apart from the liver, where their purpose can be quite different.

##### **1.4.2.2.1 Carbamoyl Phosphate Synthase I**

There are two forms of Carbamoyl Phosphate Synthase (CPS), CPSI is a hepatic mitochondrial enzyme involved in the urea cycle and found mainly in the liver and intestine, and CPSII, a cytoplasmic enzyme involved in pyrimidine biosynthesis. CPSI is the rate-limiting enzyme of the urea cycle (66). The human CPSI gene is localised to chromosome 2q35 that spans ~122kb and encodes a 5.7kb mRNA sequence (NM\_001875) (67). The protein is a dimer composed of 160kDa subunits. It is

responsible for the first committed step in the urea cycle and is the most abundant enzyme; at 0.4-0.7mM it can constitute as much as 30% of mitochondrial protein and 4% of total cell protein in the liver as it is highly stable. The N-terminus of CPSI contains a 38 amino acid leader peptide that directs it to the mitochondria. This is a highly basic region rich in arginine, lysine and aspartate residues. These properties are common to many mitochondrial proteins, with the leader peptide directing the precursor molecule into the mitochondria, which is then cleaved to leave a protein that can be folded into the correct conformation in the mitochondria (21;68). Once ammonia is released from glutamine or glutamate, it immediately enters the CPSI binding site since CPSI and glutaminase are both activated simultaneously by NAG, and are also in very close proximity (66;69).

#### **1.4.2.2.2 Ornithine Transcarbamylase**

Ornithine Transcarbamylase (OTC) is a mitochondrial enzyme that is located mainly in the liver and in smaller amounts in the kidney and intestine. It catalyses the second step of the urea cycle. The OTC gene is located on the X-chromosome (70) at Xp21.1, spans ~85kb and contains 10 exons. These encode a 1.9kb mRNA that contains ~500bp of untranslated region, and a 1kb coding sequence (NM\_000531). The first exon contains a 32 codon mitochondrial leader peptide. This is located at the N-terminus and is arginine rich but contains no acidic residues. The protein is a homotrimer composed of 36kDa subunits. The tissue specific expression of OTC is tightly controlled and activity is rapidly lost in cultured hepatocytes and is at minimal levels in hepatoma cell lines (21;71-74).

#### **1.4.2.2.3 Argininosuccinate Synthetase**

Argininosuccinate Synthetase (AS) is a cytosolic enzyme found in the liver, kidney, fibroblasts and in trace amounts in the brain. It is involved in the third step of the urea cycle. The gene is located at chromosome 9q34 and spans ~63kb with 16 exons that encode a ~1.2kb coding sequence (NM\_000050). This encodes a homotetrameric protein of 46.4kDa. There are a number of copies of the AS gene scattered throughout the genome but the only functional protein is encoded for by the sequence on chromosome 9. Two transcript variants exist of this AS gene, they differ in the 5' untranslated region but both encode the same protein (21).

#### **1.4.2.2.4 Argininosuccinate Lyase**

Argininosuccinate Lyase (AL) is also a cytosolic enzyme found in the liver, kidney, brain and fibroblasts. It catalyses the fourth step of the urea cycle. The gene is located on chromosome 7q11.2 and spans ~26kb that encode a ~1.3kb mRNA sequence (NM\_000048). The protein is a tetramer of 52kDa subunits (21).

#### **1.4.2.2.5 Arginase I**

Arginase exists in two forms, arginase I and arginase II. Arginase II is a mitochondrial enzyme that is found in small amounts in the kidney, intestine and brain. Arginase I is a cytosolic enzyme found in the liver and erythrocytes, and is responsible for processing the final step of the urea cycle. The hepatic arginase I gene is located at chromosome 6q23. The gene spans 11.5kb and contains 8 exons that encode a 1.5kb mRNA product (NM\_000045). The protein is a homotrimer composed of 35kDa subunits.

#### **1.4.2.3 Urea Cycle Enzymes in Other Roles**

It has been proposed that in some tissue types or during some disease states, the functions of CPSI may overlap with those of CPSII, that is to provide a supply of carbamoyl phosphate for pyrimidine biosynthesis, although this is still unclear (75-77). CPSI, together with OTC, are also expressed in the intestine, where they act together to produce citrulline. This is then transported to the kidney, where another two urea cycle enzymes are expressed. AS and AL convert the citrulline to arginine, which is then released into the circulation. These two enzymes have another important role to play in general cell function, they are involved in nitric oxide (NO) synthesis (78). NO is made from the break-down of arginine to citrulline by NO synthase, of which a number of forms exist. The citrulline is converted back to arginine by AS and AL. NO levels can change dramatically in various disease states including infection and cancer (78). Arginase I is a liver-expressed enzyme, whilst Arginase II, a 38kDa protein, shares a high degree of homology with type I, and contains a 32 amino acid mitochondrial leader peptide at the amino terminus. The activity of arginase II is the same as that of arginase I, in that it also converts arginine to ornithine. However, it is highly expressed in the mitochondria of the kidney, brain, intestine, and prostate. The role of arginase II is unclear, however it has been implicated in regulating nitric oxide synthesis and playing a role in polyamine metabolism, and glutamate and proline synthesis (21;79).

## 1.5 Summary

This introduction highlights the importance of ammonium detoxification and the role hepatocytes play in maintaining low circulating ammonia by rapidly altering urea cycle expression in response to levels of nitrogenous waste. Since liver failure patients are unable to efficiently carry out these functions, ammonia must be removed effectively by BAL devices in a suitable way. Currently, the biological component of these devices have limitations in this regard. Human hepatocytes would provide the ideal cell type for BAL devices however they are not at present a viable option. In order to address this issue, investigators have explored the use of porcine hepatocytes and immortalised human cell lines. Porcine hepatocytes exhibit good hepatocyte function, but zoonoses are a potential problem. Immortalised human cell lines exhibit good hepatocyte synthetic functions but have been shown to lack full detoxificatory capabilities. In addition, there is a risk of oncogenic transformation using these cell lines.

A number of systems have been developed and optimised using immortalised human cell lines, including our own, which uses HepG2 cells as the biological component of the BAL device. The expression of hepatocyte specific functions by these cells has been improved considerably by their encapsulation in alginate and culturing them in this 3D configuration. Despite this, detoxificatory capacity is still limited, with the production of urea via urea cycle, the mechanism of ammonia removal, not evident in this cell line.

This thesis investigates and characterizes the lack of urea cycle function in HepG2 cells and offers and tests solutions for the restoration of the cycle and increased ammonia detoxification. In addition, urea cycle function in the HepG2-derived C3A cell line was studied, as these cells are reported to produce urea, and could offer a suitable alternative to HepG2 cells.

## **1.6 Hypothesis and Aims**

The aims of the following research lie in the context of developing a cell source suitable for use in a BAL that has the ability to detoxify ammonia via the urea cycle. It was hypothesized that like many cancer cells, the HepG2 cell line had down-regulated certain functions deemed unnecessary to cell survival. Therefore, there could be a mechanism by which the urea cycle was silenced and this could possibly be manipulated and reversed. One other approach could be to restore genes missing from the cycle and replenish the missing enzymes to complete the urea cycle.

The aims of the project were to:

- 1) Characterize urea cycle function in HepG2 and C3A cells.**
- 2) Restore urea cycle function in the HepG2 cell line.**

## Chapter 2: General Methodology

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The methods listed below appear throughout the project. When these methods are referred to in subsequent chapters, the basic methodology is found in this chapter, however, any modifications made to these standard protocols will appear in the methods section specific to that chapter. Reagents are from Sigma unless otherwise stated.

### 2.1 Mammalian Cell Culture

#### 2.1.1 Monolayer Culture of HepG2 and C3A cells

##### *Materials*

HepG2 cell line (ECACC, Wiltshire UK)

C3A cell line (LGC Promochem, Middlesex, UK)

$\alpha$ -MEM with ribonucleosides and deoxyribonucleosides (Gibco #22571, Invitrogen)

MEM with Glutamine and Earles Salts (Gibco #31095-029, Invitrogen)

Sodium pyruvate

Non-essential amino acids

Hanks Buffered Salt Solution without calcium or magnesium (HBSS) (Gibco, Invitrogen)

Trypsin (Citrate Saline pH7.8 (4.4g Trisodium citrate, 10g KCl in 1L water), autoclave.

Filter sterilise 500mg glucose (dissolved in a little citrate saline) into the saline buffer.

Add 25ml of 10x Trypsin/EDTA from Gibco to give 0.125% final trypsin solution.)

2% Trypan Blue in PBS

5ml, 10ml, 20ml, 50ml Syringes

200 $\mu$ m filters

21G needles

Tissue culture flasks

Supplements, see Table 2.1 (page 45)

Centrifuge

##### *Method*

To maintain HepG2 cells in monolayer culture, they were seeded at a density of  $0.5 \times 10^6$  cells in 30ml for a T175 flask to reach confluency of 70-80% in a week and to yield 25-30 million cells. The cells were maintained in an incubator at 37°C in a humidified atmosphere of 95 % air and 5 % CO<sub>2</sub>. Medium was changed every 2 days. Medium, trypsin and HBSS were warmed prior

to use. The medium was made by filter sterilizing the supplements in the  $\alpha$ -MEM (referred to as complete medium). C3A cells were cultured in MEM with glutamine and Earle's salts with 10% FCS, 1mM sodium pyruvate and 0.1mM non-essential amino acids, and 50U/ml pen/strep and maintained as for HepG2 cells.

*Table 2.1: Supplements added to  $\alpha$ -MEM culture medium.*

<b>Supplement</b>	<b>Final concentration in 500ml</b>
Foetal Calf Serum (FCS) (Hyclone)	10%
Penicillin/Streptomycin (Gibco, Invitrogen)	50U/ml
Fungizone (Gibco, Invitrogen)	0.292g/L
Glutamine (Gibco, Invitrogen)	1.25 $\mu$ g/ml
Linoleic acid albumin	0.05mg/ml
Hydrocortisone	0.04 $\mu$ g/ml
Thyroid Releasing Hormone	0.04 $\mu$ g/ml
Insulin (Nova Nordisk)	0.30U/ml
Sodium selenite	0.002 $\mu$ g/ml

When cells reached 80% confluency, the cells were split by pouring off the culture medium, washing the cells with 10ml warm HBSS three times and incubating the cells with 10ml trypsin at 37°C for 1-2 minutes. The flask was tapped firmly so that the cells detached from the surface. 30ml warm complete medium was added to stop the action of the trypsin. The cells were centrifuged at 300g (1200rpm) at room temperature for 4 minutes. The supernatant was discarded and the cells resuspended in 5ml complete medium. The cells were then syringed with a 21G needle 4-5 times to disaggregate cell clumps. To determine cell number and viability, trypan blue exclusion was used. Trypan blue stains dead or damaged cells as intact membranes exclude trypan blue. This was done by mixing 160 $\mu$ l of HBSS, 20 $\mu$ l of trypan Blue and 20 $\mu$ l of homogenous cell suspension. After 2 minutes 10 $\mu$ l of the solution was loaded onto a haemocytometer chamber and viable and non viable cells were counted. Once cell number was determined, the cell suspension was syringed 2-3 more times prior to reseeding at the appropriate density.

### **2.1.2 Alginate Encapsulation and 3D Culture**

Cells were encapsulated by colleagues more experienced in using the equipment.

#### *Materials*

Clean baked Pyrex beakers

Plastic beaker for collecting waste from Inotech machine



Plastic bottomless beaker and rubber band  
200µm nylon mesh  
Stainless steel forceps  
Magnetic stirrer  
50µm syringe filter  
Spatula  
6-well plates containing 200µm cell strainers  
Sterile 2% alginate (Alginic acid) in 0.15M NaCl solution  
Polymerisation buffer (0.15M NaCl, 0.204M CaCl<sub>2</sub>, 0.05% Pluronic acid)  
Complete medium (500ml) with glucose added, 4.44ml of a 45% D-glucose solution (referred to as HG (high glucose) medium)  
DMEM medium (Gibco #31331, Invitrogen), supplemented with 10% FCS, Pen/Strep and Fungizone (concentrations as for α-MEM)  
15ml and 50ml tubes  
50ml syringes  
25 ml serological pipettes  
T175 Culture flasks  
Inotech Cell Encapsulator

### *Methods*

The day prior to the experiment 2% w/v alginate was prepared and stirred overnight in the dark. The next day the mixture was autoclaved at 121°C for 10 minutes and left to cool. Cell strainers were clipped and placed in six well plates. Cells were trypsinized according to the previously described method and the pellet resuspended in 5ml of HG medium; cell number and viability was determined. For the 3D culture, a set volume of alginate/medium mixture is run through the encapsulator. Twice as much as the final volume of beads required was prepared; this volume is referred to as  $y$ . An aliquot of HepG2 cells was added to a 50ml Nunc tube containing  $(y/2) \times 10^6$  HepG2 cells. The volume is made up to  $y$  using a 1:1 mixture of 2% alginate and HG medium and gently mixed. The cell/alginate/medium mixture was drawn into a 50ml syringe through the 50µm filter. The syringe containing alginate/cell mixture was attached to the inlet port of the encapsulator and placed on the syringe pump (Figure 2.1). A pyrex beaker containing a flea, and filled with 300ml polymerisation buffer was placed on a magnetic stirrer directly below the nozzle. The stirring speed was set to the lowest level possible to maintain constant rotation of the flea. The stainless steel forceps were placed into the Pyrex beaker with one arm on either side of the beaker wall. The crocodile clip was connected with the black wire to the forceps to earth the beaker and polymerisation buffer. The waste collection cup was placed below the nozzle and the vibration unit switched on followed by the syringe pump. The flow rate was set at 5ml/minute. Once the turbo button was

released, the electrostatic dispersion ring was moved into place so that the liquid stream passed through its centre. The current was switched on to the electrostatic dispersal unit and the stream of beads allowed to stabilise; the collection of beads was continued until 5ml of liquid was left in the syringe. At this point the waste collection cup was replaced under the stream and the syringe pump, vibration unit and electrostatic dispersal unit switched off. The beaker containing the beads was covered and left to stand for 5 minutes with stirring, to allow for polymerisation of the alginate (Figure 2.1).

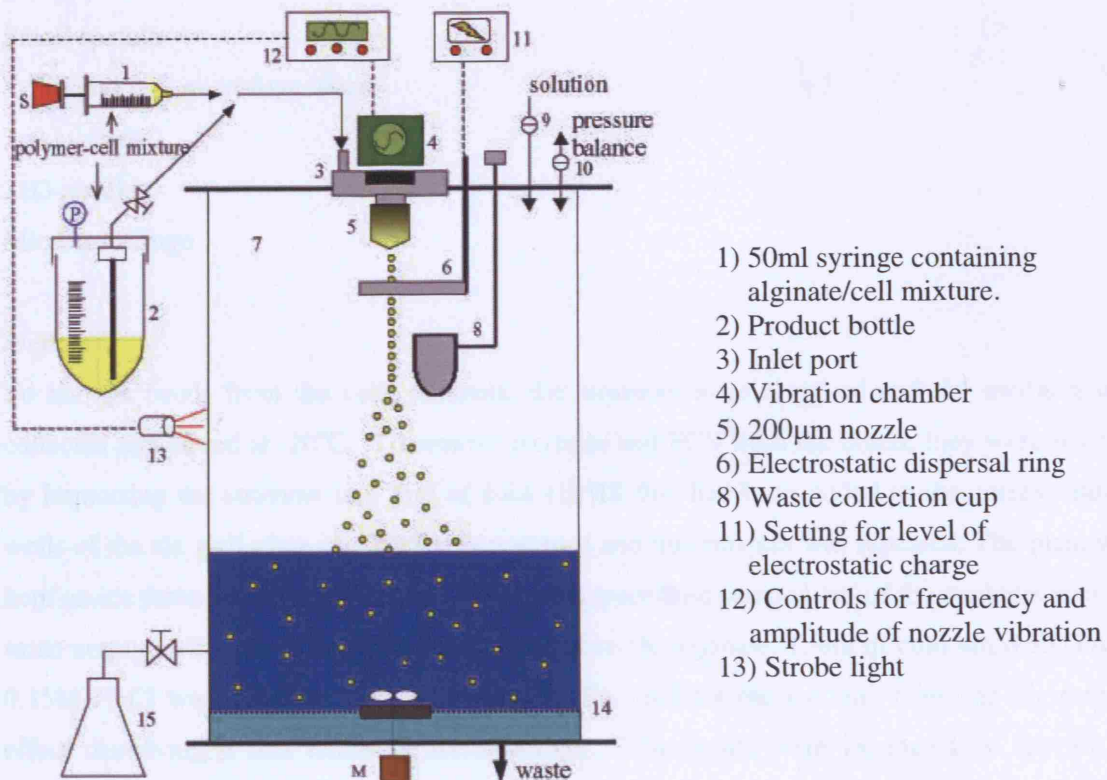


Figure 2.1: Diagram of the Inotech machine and the production and collection of alginate beads.

### Collecting, washing and plating the beads

The beads were removed from the polymerisation buffer by pouring them into a beaker with a 200µm nylon mesh bottom. The beads were rinsed with 60ml DMEM before placing the beaker and mesh in a baked beaker and adding DMEM until the beads were just covered. The beads were transferred into a 15ml centrifuge tube, resuspended in HG medium and allowed to settle. The volume of beads collected was estimated and the beads resuspended in a T175 tissue culture flask with HG medium at a ratio of 0.25ml beads:8ml culture medium. This collection was then thoroughly resuspended to be aliquotted as 8ml into each cell strainer in the

6-well culture plates. Plates were kept in an incubator maintained at 37°C and 5% CO<sub>2</sub>. The culture medium was changed every 2 days.

### **2.1.2.1 Removing Cells from Alginate**

#### *Materials*

1xPBS

4mM EDTA in 0.15M NaCl, pH7.4

0.15M NaCl, pH7.4

6-well plates

Small spatula

1.5ml and 2ml microfuge tubes

2ml syringe

21G needles

Microcentrifuge

#### *Methods*

To harvest beads from the cells strainers, the strainers were removed and the medium was collected and stored at -20°C. To remove medium and FCS from the beads, they were washed by immersing the strainers into 5ml of cold 1xPBS that had been added to the corresponding wells of the six well plate, the PBS was removed and this process was repeated. The plate was kept on ice throughout the procedure. The beads were then scraped out of the strainers into the same empty well using a small spatula. To remove the alginate, 1.8ml of cold 4mM EDTA in 0.15M NaCl was added to each well. The EDTA chelates the calcium from the alginate in effect dissolving it and releasing the spheroids. The beads were incubated on ice for 20 minutes. Using a 2ml syringe and a 21G needle, the spheroid solution was transferred to a 2ml microfuge tube and syringed 4-5 times to disaggregate the spheroids. The samples were centrifuged at 10000xg for 5 minutes at 4°C. The supernatant was removed and the pellet washed with 1.5ml of 0.15M NaCl and centrifuged again. All the supernatant was removed leaving the cell pellet to be used for subsequent experiments.

### **2.1.3 Primary Human Hepatocyte Culture**

#### **2.1.3.1 Isolation of Primary Human Hepatocytes**

This was carried out by other colleagues. We were authorised to use this tissue.

#### *Materials*

Chelating Buffer (20mM HEPES, 0.5mM EGTA in PBS)

Perfusion Buffer (20mM HEPES in PBS)

Digestion Buffer (20mM HEPES, 1.5g BSA (0.5% w/v), 50µg/ml ascorbic acid and 4µg/ml insulin in 300ml HBSS. Just prior to digestion of the liver 150mg Collagenase Type IV (0.05% w/v) and 30mg DNase I (0.01% w/v) added)

Dispersal Buffer (50ml (10%v/v) FCS and 50mg DNase I (0.01% w/v) to 500ml Williams E medium)

Collagenase (Sigma)

### *Method*

Primary human hepatocytes were isolated from a freshly resected piece of human liver by collagenase digestion and differential centrifugation (80). Hepatocyte cultures were pure for hepatocytes as observed using a microscope. After extended culture (>7 days), fibroblasts did grow, however in the following experiments hepatocytes were used within 48 hours.

### **2.1.3.2 Collagen Coating Plates**

#### *Materials*

6-well plates

12-well plates

Sterile HBSS, calcium and magnesium free

Sterile Saline

Collagen type I prepared from rat tail tendons (1g tendons in 300mls 0.01M sterile acetic acid, stirred at 4°C for 2 days, centrifuged at 800xg for 2 hours and stored at 4°C)

UV lamp

#### *Method*

To a 12-well plate and to a 6-well plate, 1ml/well or 2ml/well of collagen was added, respectively. After 5 minutes the collagen was flicked vigorously out of the wells and the wells rinsed twice with 2ml or 4 ml HBSS. Then 1ml or 2mls of sterile saline was added to each well of a 12-well or 6-well plate respectively and the plate irradiated under short wave UV light for 15 minutes, without the lid. The plates were used on the same day.

### **2.1.3.3 Culture of Primary Human Hepatocytes**

#### *Materials*

Williams E Medium (Gibco #22551, Invitrogen)

10%FCS

50U/ml Penicillin/Streptomycin

0.292g/L Fungizone

1.25µg/ml L-glutamine

10<sup>-8</sup>M Insulin

20ng/ml Hepatocyte Growth Factor (HGF)

10ng/ml Epidermal Growth Factor (EGF)

#### *Method*

Williams E medium was made up with all the above supplements (final concentration concentrations listed) except for the HGF and EGF. Once isolated, hepatocytes were seeded in collagen coated plates at 1x10<sup>6</sup>cells/ml, with 2ml per well of a 6-well plate and 1ml per well of a 12 well plate. The following morning the medium was aspirated and the cells washed twice with 1ml of non-supplemented Williams E medium. The medium was then replaced with Williams E supplemented with or without HGF and EGF at the above concentrations. The medium was then changed every 24 hours after that, with or without HGF and EGF supplementation.

#### **2.1.4 Nuclei Count**

##### *Materials*

1xPBS

Nucleocounter and accompanying reagents (Chemometec)

1.5ml microfuge tubes

##### *Methods*

Fresh cell pellets obtained after removing cells from alginate were resuspended in 200µl 1xPBS. An equal volume of lysis reagent A was added and the samples vortexed, then, an equal volume of stop solution B was added and the samples vortexed. The nucleocounter cassette was loaded with cell lysate and placed into the nucleocounter to take a count of viable cells.

#### **2.1.5 Bradford Reagent Assay**

##### *Materials:*

Coomassie Plus-200 Protein Assay Reagent (Perbio)

2mg/ml Bovine Serum Albumin (BSA)

Multipipettor

96 well plate (Nunclon)

Dynex Spectrophotometer at 595nm

Revelation Plate Reader software

#### *Method:*

A standard curve was prepared ranging from 20µg/ml to 1µg/ml BSA. 100µl of standard or test sample were loaded into the 96 well plate in triplicate. An equal volume of Coomassie reagent was then added using a multipipettor and the plate read on the spectrophotometer at 595nm, ensuring that there were no bubbles on the surface of the wells that interfere with the reading, (81).

### **2.1.6 Bicinchoninic Acid Protein Assay**

The Bicinchoninic Acid (BCA) assay was carried out on samples which had been lysed in detergent as the assay is not as easily affected by detergents, unlike the Bradford assay, (82).

#### *Materials:*

Bicinchoninic Acid (BCA)

BCA reagent (1% w/v BCA, 2%Na<sub>2</sub>CO<sub>3</sub>, 0.16% NaK tartrate, 0.4% NaOH, 0.95% NaHCO<sub>3</sub>, pH 11.25 with 50% NaOH)

4% w/v Copper Sulphate

10mg/ml Bovine Serum Albumin

Plate sealers

Plate shaker

96-well plate

Spectrophotometer at 570nm

#### *Method:*

A standard curve was prepared from BSA with the highest standard 2mg/ml. The BCA reagent and 4% copper sulphate were prepared according to manufacturers' instructions. Immediately prior to the assay BCA and copper sulphate were mixed in the ratio 50:1. 200µl of this working reagent was added per well of the 96 well plate. 10µl of standard or sample was then added, and these mixed on a plate shaker. The plate was sealed and incubated at 37°C for 1 hour. A reading was taken on the spectrophotometer at 570nm.

## **2.2 Molecular Methods**

### **2.2.1 RNA Extraction and Quantification**

#### *Materials*

RNagents Total RNA Isolation Kit (Promega)

1xPBS, autoclaved  
75% ethanol, ice cold  
1.5ml microfuge tubes, autoclaved  
Microcentrifuge, 4°C  
Vacuum desiccator  
NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies)

### *Methods*

The protocol was followed according to manufacturers' instructions. To prepare RNA from fresh hepatocytes and cells from 3D culture, once the cells were isolated they were washed twice with cold PBS and centrifuged at 1000xg in a 1.5ml microfuge tube. The PBS was aspirated and the cells resuspended in the appropriate amount of denaturing solution, according to cell concentration, day 9 3D bead cultures were resuspended in 300µl. For monolayer culture, cells grown in a 6-well plate were washed with 1ml of cold PBS twice, the PBS removed and 300µl of denaturing solution added into the well. The mixture was pipetted a few times to gather cells and then transferred to a cold microfuge tube. To the cell suspension, 10% volume of supplied sodium acetate was added and the tubes inverted 5 times. An equal volume of supplied phenol:chloroform:isoamyl alcohol was added, the tubes inverted 5 times gently then shaken vigorously for 10 seconds and incubated on ice for 15 minutes. After this time, the tubes were centrifuged at 10000xg for 20 minutes at 4°C. The top layer was transferred to a new microfuge tube with care not to disturb the interphase material. An equal volume of supplied isopropanol was added and the RNA precipitated at -20°C for 30 minutes. The RNA was pelleted by centrifugation at 10000xg for 10 minutes at 4°C. The supernatant was removed and the pellet resuspended in ice cold 75% ethanol and centrifuged once more. The ethanol was removed and the RNA dried in a vacuum desiccator for 5-15 minutes, resuspended in 30µl of supplied nuclease-free water, quantified using the NanoDrop, aliquotted and stored at -80°C.

### **2.2.2 cDNA Synthesis**

#### *Materials*

RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Science #K1622)  
0.2ml PCR tubes  
PCR block for incubations  
Microcentrifuge, 4°C

#### *Method*

The method was followed according to manufacturers' protocol. 1µg RNA and 1µl provided random hexamer primer were added to a 0.2ml PCR tube and made to 12µl with supplied

DEPC treated water. This was mixed gently, incubated at 70°C for 5 minutes, chilled on ice and centrifuged for 5 seconds. The tubes were placed on ice and to each tube the following were added in order, 4µl 5x reaction buffer, 1µl RiboLock™ Ribonuclease Inhibitor and 2µl 10mM provided dNTP mix, mixed gently and centrifuged for 5 seconds. The tubes were incubated at 25°C for 5 minutes, then 1µl RevertAid™ M-MuLV Reverse Transcriptase added and the tubes incubated at 25°C for 10 minutes, 42°C for 60 minutes, 70°C for 10 minutes (to stop the reaction) and then chilled on ice.

### 2.2.3 Reverse Transcriptase-PCR

#### *Materials*

HotstarTaq Polymerase (Qiagen)

Oligonucleotides, stock 100µM in 15mM Tris Cl (synthesized by SigmaGenosys)

Sterile ddH<sub>2</sub>O

0.2ml PCR tubes

1.5ml microfuge tubes

PCR Block

#### *Method*

HotstarTaq polymerase requires activation at 95°C for 15 minutes, therefore it is more stable than standard Taq polymerase. The primers were diluted 1 in 10 in RNase DNase free water to give a working solution of 10µM, then the following were assembled in a 1.5ml microfuge tube, to make a mastermix that could then be aliquotted into 19µl per 0.2ml PCR tubes. The cDNA was added last to each tube (Table 2.2). The cycling parameters alter depending on the primer sequence and these are specified in the relevant chapters.

*Table 2.2: Mixture for PCR reaction.*

<b>Mastermix</b>	<b>One reaction, µl</b>
HotstarTaq	10
Primer forward	1
Primer reverse	1
Sterile ddH <sub>2</sub> O	7
cDNA	1
<b>Total µl</b>	<b>20</b>

### 2.2.4 Agarose Gel Electrophoresis

#### *Materials*

Agarose (Invitrogen)

50xTAE stock (1L in ddH<sub>2</sub>O, 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA in ddH<sub>2</sub>O, pH 8.0)



Ethidium Bromide 10mg/ml stock (stored at 4°C in the dark)

5x Loading Dye (Bioline)

Hyperladder I (Bioline)

Gel Electrophoresis Tank (BioRad)

Microwave

### *Method*

The density of the agarose gel used depended upon the size of fragments that were separated. As an example, and the most frequently used gel that appears in subsequent methods is a 1% agarose gel. 0.5g of agarose was dissolved in 50ml 1xTAE using in a microwave. When the agarose had cooled to ~60°C, 1µl ethidium bromide was added and the gel poured into the casting tray and left to solidify. The gel was immersed into the gel electrophoresis tank filled with 1xTAE and the wells of the gel flushed gently with TAE. 5µl DNA Hyperladder I was used for reference. PCR samples were mixed with 5x loading dye (1x final) and loaded into the wells. The gel was run for 45 minutes at 90V 140 Amps at constant voltage.

### **2.2.5 Real Time RT-PCR**

Real Time RT-PCR is a method by which gene expression can be quantified by incorporating a fluorescent probe into the DNA that is amplified. Therefore during every round of replication, the resulting copy number can be visualised in graphical form. A standard curve can be generated for each particular gene of interest, as explained below, and the copy number of the gene in the sample can then be calculated. Prior to real time, the oligonucleotides were tested by normal PCR to check that they bind to their target (using the appropriate positive control, usually human liver cDNA).

### *Materials*

HepG2, C3A, Primary Human Hepatocytes and Human Liver cDNA

HotstarTaq Polymerase

Oligonucleotides (as above)

Gel Extraction Kit (Qiagen)

Nanodrop (for RNA and DNA quantification)

Sybr Green (1:3000 dilution of stock in DMSO) (Biogene)

0.1ml PCR tubes and caps (Corbett Research)

RotorGene 3000 Thermal Cycler (Corbett Research)

### *Methods*

Firstly, primers were designed using Vector NTI software to amplify gene specific fragments of less than 200bp. Sequences for primers used in each chapter are listed with the cycling

conditions in the relevant sections. To create the standard curves used to quantify gene expression, PCR was carried out on human liver cDNA using each set of primers (sequences are listed in relevant chapters). The product of the PCRs was run on an agarose gel, the band excised, and cDNA extracted using a gel extraction kit according to the manufacturers' protocol. The cDNA was quantified using the NanoDrop Spectrophotometer. Using the concentration of DNA and the fragment length created by the primers, it is possible to theoretically determine the number of molecules per  $\mu\text{l}$  of cDNA. The calculation is as follows:

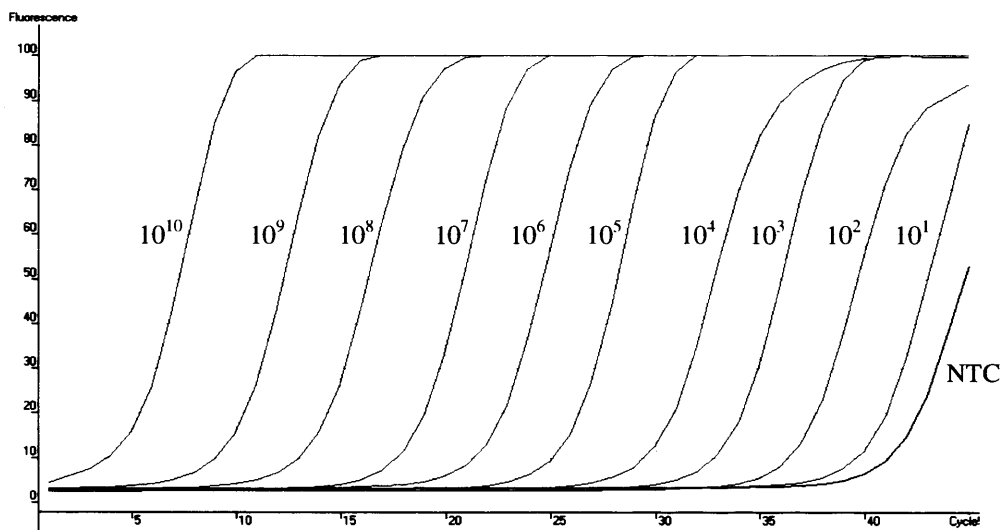
$330 \times \text{length of amplicon} = \text{Molecular weight (MW) of amplicon}$

$\text{MWg} = \text{Avogadro's number } 6.022 \times 10^{23}$

$\text{Amplicon molecules/ng} = 6.022 \times 10^{14} / \text{amplicon MW}$

$\text{Concentration of amplicon from gel excision ng/}\mu\text{l} = \text{Amplicon molecules/}\mu\text{l}$

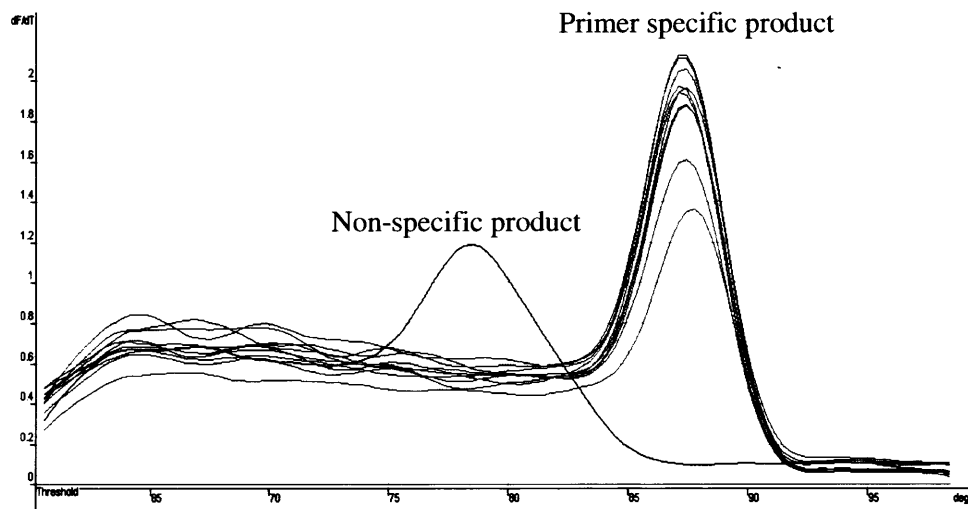
The following dilutions were made for the standard curve:  $10^{10}$ ,  $10^9$ ,  $10^8$ ,  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$  and  $10^1$  molecules per  $\mu\text{l}$ . One example of a standard curve generated is seen in Figure 2.2.



*Figure 2.2: Standard curve generated during Real time RT-PCR with a no template control (NTC) that tends to appear after 40 cycles.*

Real Time RT-PCR was carried out by, firstly, diluting the  $10\mu\text{M}$  stock of primers by adding forward primer, reverse primer and water in the ratio 1:1:2, secondly diluting the Sybr Green 1:3 in water, and then thirdly assembling the mastermix by adding  $10\mu\text{l}$  HotstarTaq polymerase mix,  $5.1\mu\text{l}$  water,  $2.4\mu\text{l}$  diluted primers,  $1.5\mu\text{l}$  diluted Sybr green. The mix was aliquotted,  $19\mu\text{l}$  into  $0.1\text{ml}$  tubes, and lastly  $1\mu\text{l}$  standard or sample cDNA added per  $20\mu\text{l}$  reaction.

The cycling conditions include a polymerase activation step 95°C for 15minutes, and a denaturing, annealing and elongation temperature specific to each gene (conditions specified in relevant chapters). A melt curve, where the products of the PCR are separated by increasing the temperature following the end of the PCR, indicates the specificity of the primers to generate of a specific product, as seen in Figure 2.3 (page 56); the non-matching peak shows the presence of a non-specific product. The melt curve analysis is equivalent to running the PCR products on a gel, where the only visible band should correspond to the amplified product.



*Figure 2.3: Melt curve analysis. Overlapping peaks for the primer specific products created for each standard; the lone peak is an example of a non-specific product.*

### 2.2.6 Statistical Analysis

Statistical analysis was carried out on data sets using GraphPad Prism software to perform Student's unpaired t-tests and to determine significance values. In each figure legend, the number of samples used in one experiment is denoted by n with mean $\pm$ SD calculated for the graphs. Also listed in the figure legends in the number of times the experiment was repeated. The graph displayed is of one representative experiment.

### **Chapter 3: Characterizing Urea Cycle Function in HepG2 and C3A Cells**

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Human liver-derived cell lines are widely used as surrogates for primary human hepatocytes as they confer many hepatocyte-specific characteristics, both synthetic and detoxificatory (83). Two such cell lines, the hepatoblastoma-derived HepG2 cell line and the HepG2 subclone C3A, are commonly used as such (84). Both the HepG2 and C3A cell lines are currently being considered for their potential suitability as the cellular component of a BAL, with one system comprising C3A cells, a subclone of HepG2 cells, entering clinical trials (37;38). These cell lines do exhibit significant hepatocyte-like functions, however they have lost some of their synthetic and detoxificatory capacity and therefore lack the full repertoire of liver-like functions required of a BAL (33;34).

One key detoxificatory function required of such BAL systems is the provision of ammonia detoxification. Patients with acute liver failure have compromised ammonia detoxification capabilities leading to elevated ammonia concentrations and glutamine production, which can result in inducing hyperammonaemia and subsequently hepatic encephalopathy (43-46). In normal hepatocytes, ammonia is removed via the urea cycle (Figure 3.1). In HepG2 cells however, this function is not apparent despite an up-regulation in hepatocyte specific functions when HepG2 cells are grown in 3D culture (40;85). In comparison, the C3A cell line, a subclone of the HepG2 cell line, is reported to produce urea, a finding which has been believed to indicate the presence of a fully functional urea cycle capable of ammonia detoxification via this specific pathway (38;86-89).

The aims therefore of Chapter 3 were to elucidate the reasons behind the lack of the urea cycle function in HepG2 cells, which were not yet identified, and to determine the extent of urea cycle function in C3A cells to assess whether they could offer a suitable replacement, as regards ammonia detoxification and urea production, for the HepG2 cell line.

To characterise urea cycle function in HepG2 and C3A cells the following were investigated:

1. **Urea synthesis:** this was investigated by using a colorimetric urea nitrogen assay. However, as this method was too insensitive Gas Chromatography-Mass Spectrometry (GC-MS) was used to detect urea production. Metabolic labelling studies were then carried out to determine the level of urea cycle activity using  $^{14}\text{C}$ - $\text{NaHCO}_3$  labelling and Thin Layer Chromatography (TLC) to separate urea and the urea cycle intermediates citrulline and arginine. To then specifically investigate ammonia detoxification, the incorporation of  $^{15}\text{NH}_4\text{Cl}$  into  $^{15}\text{N}$ -labelled urea was determined.
2. **Urea cycle gene expression:** mRNA expression levels of the urea cycle genes, CPSI, OTC, AS, AL and Arginase I were determined by Real Time Reverse Transcriptase-PCR (Real Time RT-PCR).
3. **Urea cycle enzyme expression:** enzyme assays were carried out to confirm whether gene expression results were reflected by functional enzyme. CPSI and OTC combined activity was determined by labelling cell lysates with  $^{14}\text{C}$ - $\text{NaHCO}_3$  and  $^3\text{H}$ -labelled ornithine, which would give  $^{14}\text{C}^3\text{H}$ -citrulline from functional CPSI and OTC. AS and AL activity were also assessed together by labelling with  $^{14}\text{C}$ -labelled citrulline to give  $^{14}\text{C}$ -arginine. Arginase activity was determined by the conversion of  $^{14}\text{C}$ -arginine to  $^{14}\text{C}$ -urea.

This next section summarises the techniques used to characterize the extent and degree of urea cycle activity in HepG2 and C3A cells. Materials are from Sigma unless otherwise stated.

### 3.1 Urea Synthesis by HepG2 and C3A Cells

To determine the levels of urea synthesized by HepG2 and C3A cells, a number of assays were used. Firstly, a urea nitrogen spectrophotometric assay, which converts urea back to ammonia via NADH consumption, was used. The second method used was Gas Chromatography-Mass Spectrometry (GC-MS), which is a highly sensitive technique. Thirdly, metabolic labelling studies were used to determine the incorporation of  $^{14}\text{C}$ -labelled Sodium Bicarbonate ( $\text{NaHCO}_3$ ) through the urea cycle. Thin Layer Chromatography (TLC) was to separate  $^{14}\text{C}$ -labelled urea and  $^{14}\text{C}$ -labelled urea cycle intermediates. Lastly, in order to detect *de novo* urea production, HepG2 and C3A cells with incubated with the stable isotope  $^{15}\text{N}$ -labelled ammonium chloride ( $\text{NH}_4\text{Cl}$ ) and  $^{15}\text{N}$ -urea detected using GC-MS. The mechanism of  $^{14}\text{C}$  and  $^{15}\text{N}$  labelling are shown in Figure 3.1.

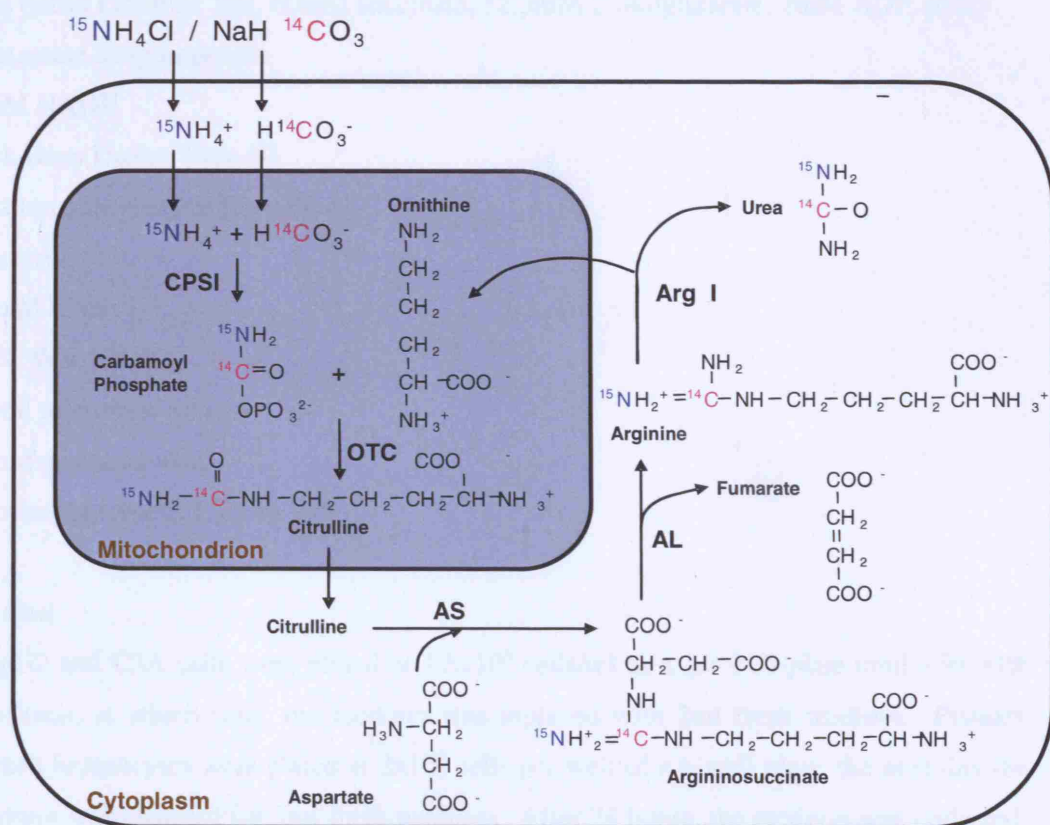
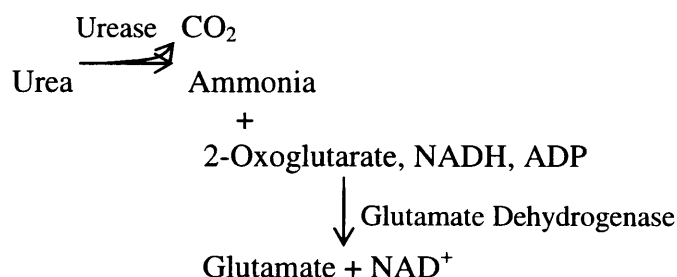


Figure 3.1: Diagram of the urea cycle. The incorporation of  $^{15}\text{NH}_4\text{Cl}$  (blue) and  $^{14}\text{C}$ - $\text{NaHCO}_3$  (pink) gives  $^{15}\text{N}$  and  $^{14}\text{C}$  labelled urea cycle intermediates and urea, respectively. CPSI: Carbamoyl Phosphate Synthetase I; OTC: Ornithine Transcarbamylase; AS: Argininosuccinate Synthetase; AL: Argininosuccinate Lyase; and ArgI: Arginase I.

### 3.1.1 Methods

#### 3.1.1.1 Urea Nitrogen Assay

The urea nitrogen assay is based on the principle of converting urea to ammonia using urease and then detecting the oxidation of NADH spectrophotometrically after the addition of glutamate dehydrogenase, as described in (90). The reaction is as follows:



#### *Materials:*

Tris buffer (150mM Tris, 60mM succinate, 12.5mM 2-oxoglutarate, 2mM ADP, pH8)

Glutamate dehydrogenase

5mM NADH

Jack Bean Urease Type III

Sodium phosphate buffer pH6.8

Glycerol

20mM Urea

25°C Water bath

1.5ml microfuge tubes

0.2ml quartz cuvette

Spectrophotometer Uvikon 930

#### *Method:*

HepG2 and C3A cells were plated at  $1.5 \times 10^4$  cells/ml in a six well plate until ~50-60% confluent, at which time, the medium was replaced with 2ml fresh medium. Primary human hepatocytes were plated at  $2 \times 10^6$  cells per well of a 6-well plate, the next day the medium was changed for 2ml fresh medium. After 24 hours, the medium was collected, lyophilised, and resuspended in 30 $\mu$ l water. The cells were harvested by scraping cells in ice-cold 1xPBS and protein quantified using the Bradford reagent assay as previously described (section 2.1.5, page 50), by sonicating cells in PBS on ice, centrifuging at 10 000g to remove debris.

For the urea nitrogen assay, the following reagents were made: (A) Tris buffer (150mM)-succinate (60mM), 2-oxoglutarate (12.5mM), ADP (2mM) buffer pH8; (B) 200 units

glutamate dehydrogenase in 5ml A; (C) 5mM NADH in A; (D) 500 units urease in 0.5 ml 20mM sodium phosphate buffer pH6.8 with 0.5 ml glycerol; and 20mM urea. They were assembled in a microfuge in order as follows: 135µl A, 10µl B, 50µl C, and 0.5µl D, then 10 µl sample. This was incubated at 25°C for 10 minutes, then the first measurement (D1) taken at 340nm, the sample was returned to 25°C for a further 15 minutes then a second measurement (D2) taken. The degree of NADH consumption ( $\Delta D$ ) was determined by  $\Delta D = D1 - D2$ , and the amount of urea determined from the standard curve created by using serial dilutions of 20mM urea stock.

### **3.1.1.2 Gas Chromatography-Mass Spectrometry to Quantify Urea and *de novo* Urea Synthesis with <sup>15</sup>N-Ammonium Chloride Labelling**

Gas Chromatography-Mass Spectrometry (GC-MS) was used since it is a highly sensitive technique with which urea can be detected after derivitizing it with dimethylformamide demethylacetal (DMF DMA). In addition, specific ammonia removal could be monitored in cells labelled with the stable isotope <sup>15</sup>N-ammonium chloride (NH<sub>4</sub>Cl). Cells capable of detoxifying ammonia via the urea cycle would take up the substrate and convert it into <sup>15</sup>N-labelled urea (Figure 3.1, page 59).

#### **3.1.1.2.1 Spiking cells with <sup>15</sup>NH<sub>4</sub>Cl and Urea Derivitization**

##### *Materials*

<sup>15</sup>NH<sub>4</sub>Cl (CK Gas Products Ltd, #NLM-467)

<sup>15</sup>N<sub>2</sub>Urea (CK Gas Products Ltd, #NLM-233)

Glass pasteur pipettes

Glass wool

Dowex 50WX8-400 ion-exchange resin (Sigma Aldrich, #21,751-4)

0.1M HCl

Acetonitrile (dried with molecular sieve)

Methanol (dried with molecular sieve)

Dimethylformamide dimethylacetal (DMF DMA)

4M NH<sub>4</sub>OH

Test tubes

Screw cap tubes, 8ml

Lyophiliser

Heating block

Vials and Caps for GC/MS (Chromacol, #03-FISV and #9-SC(B)-8RT1)

##### *Method*

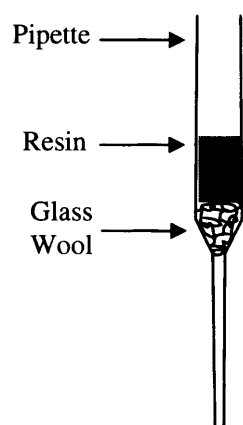
Monolayer HepG2, C3A cells and primary human hepatocytes were cultured as



previously described. For all experiments, both medium and cells were harvested at 0 hours and after 24 hours, n=6 for both time-points. For all experiments, the cells were sonicated in 400µl water as previously described, and protein quantified (section 2.1.5, page 50).

For  $^{15}\text{N}$ -labelling experiments, HepG2, C3A cells and hepatocyte cultures were set up as above. At the time of changing the medium, 1mM  $^{15}\text{NH}_4\text{Cl}$  final concentration was added. Cells and medium were harvested after 24 hours as previously described.

The conditioned media from all samples was enriched for urea by passing the media through Dowex 50WX8-400 ion exchange resin in the columns. The resin was prepared by vigorously stirring 100g in 500ml 0.1M HCl, leaving the resin to settle and pouring off as much as possible, repeated 5 times. This process was then repeated using clean water until the pH reached pH5-6. The resin was stored in water at 4°C. Ion exchange columns were made by blocking the narrow end of a glass Pasteur pipette with glass wool and adding 2cm in height of ion exchange resin (Figure 3.2). The pipette was placed into a test tube.



*Figure 3.2: Diagram of the ion exchange column.*

To prepare the samples, 15µl of 0.1M HCl was added to 0.5ml of conditioned medium sample to acidify it, followed by the addition of the internal standard, 5µl of 100mM stock  $^{15}\text{N}_2$ -urea, to give a 1mM final concentration. The samples were then loaded onto the column and after it had passed into the resin, the resin was washed with 4ml water. The pipette was then transferred to the screw cap tube and the urea eluted with 3ml 4M  $\text{NH}_4\text{OH}$ . The caps were replaced firmly and the tubes were placed almost horizontally in -80°C to freeze the eluate prior to lyophilisation. The caps were loosened just before placing in the lyophiliser

To derivitise urea, 200µl DMF DMA:acetonitrile: methanol in the ratio 3:2:1 was added to each lyophilised sample, these were heated at 70°C for 1 hour. The derivitization mixture was transferred to the GC-MS vials, these were capped and placed in the GC-MS

carousel. Values were corrected using the internal standard in each sample (1mM) to obtain  $\mu$ moles urea, then values corrected for protein or nuclei count (section 2.1.5 and 2.14 respectively, page 50).

### 3.1.1.2.2 GC-MS

#### Materials

Agilent Ultra 2 column

Agilent 6890 GC with 5973 MS (run in electron input mode)

Chemsation Software

#### Method

An Ultra 2 column was used with 50m length, 0.32mm diameter and initial flow 1.0ml/min The GC was run in splitless mode with 2 $\mu$ l injection volume. The GC profile for urea was as follows: initial temperature 200°C; rate: 5 minutes 260°C, 10 minutes 300°C. The MS detector transfer line heater initial temperature was 280°C. The MS acquisition parameters were low mass 45 and high mass 300, MS Quad 150°C maximum 200°C and MS Source 230°C maximum 250°C.

### 3.1.1.2.3 Identifying the Correct Molecular Ion

The derivitizing reagent used is DMF DMA. Two DMF DMA molecules bind to urea to make the derivitized complex detected by GC-MS (Figure 3.3). Derivitized unlabelled urea gives a mass of 170.1. Cells incubated with  $^{15}\text{NH}_4\text{Cl}$ , produce urea with one of the nitrogens labelled so gives a mass of 171.1. The internal standard used is  $^{15}\text{N}_2$ -urea and gives a mass of 172.1 as it contains two  $^{15}\text{N}$  atoms and is therefore a suitable internal control for both  $^{15}\text{NH}_4\text{Cl}$  labelled and unlabelled experiments.

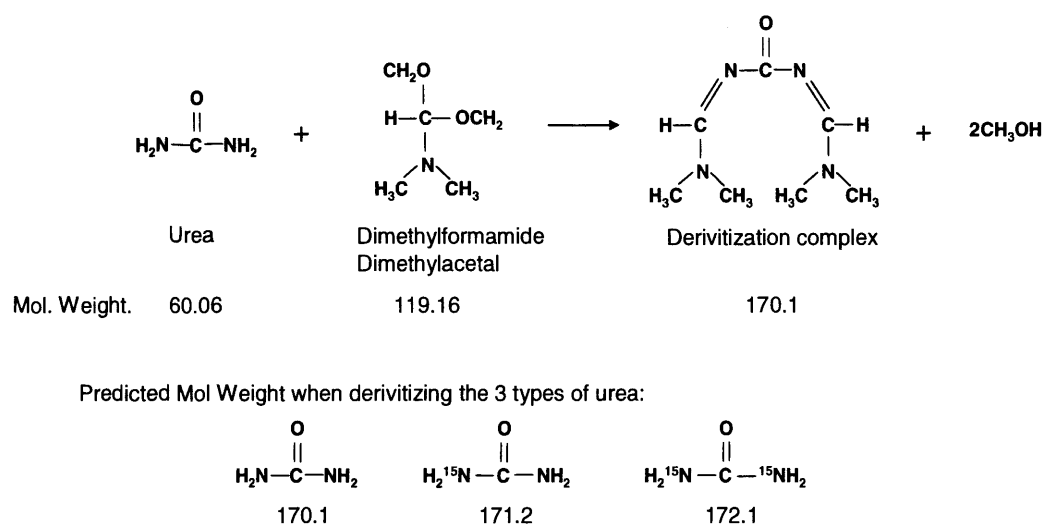


Figure 3.3: Derivitizing urea. The mechanism by which DMF DMA derivitizes urea and the expected masses with the three types of labelled urea.

All of these are detected as one product, DMF DMA-Urea by GC and the different masses by the MS (Figure 3.4).

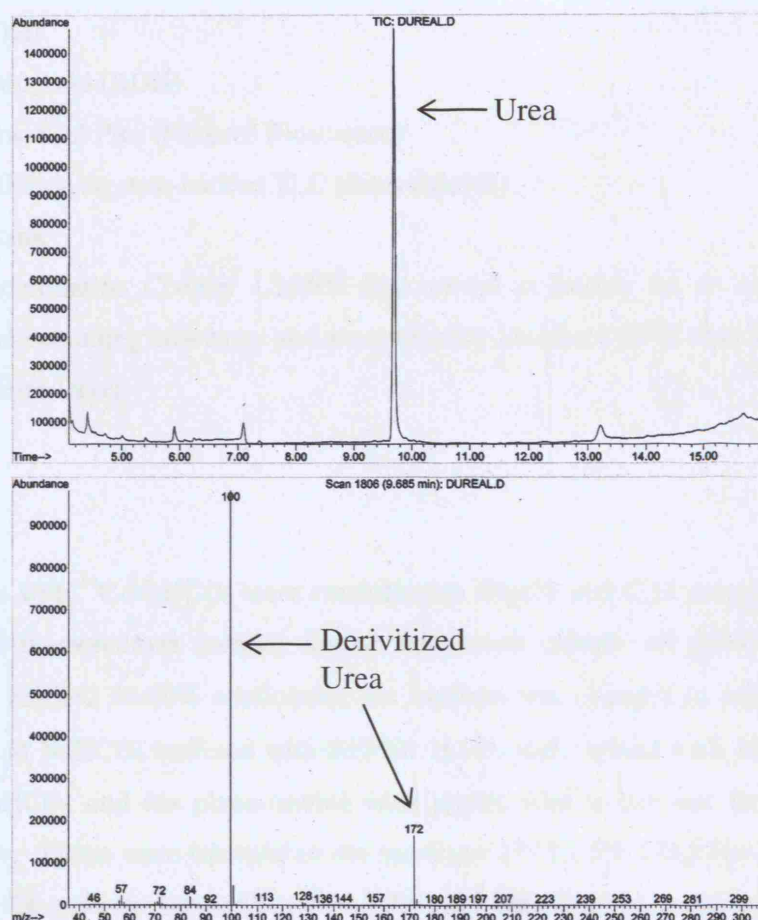


Figure 3.4: GC-MS spectra of urea. The top graph shows the peak for urea. The bottom graph shows the molecular ions detected for the  $^{15}\text{N}_2$ -urea standard.

### 3.1.1.3 Metabolic Labelling With $^{14}\text{C}$ - $\text{NaHCO}_3$ and TLC for the Detection of $^{14}\text{C}$ Labelled Urea

HepG2 and C3A cells were labelled with  $^{14}\text{C}$ - $\text{NaHCO}_3$ , which is incorporated into the urea cycle to give  $^{14}\text{C}$ -labelled urea (Figure 3.1, page 59). TLC was used separate out the  $^{14}\text{C}$ -urea, which was quantified by scintillation counting. The presence of urea was confirmed by treating samples with urease which specifically removes urea.

#### Materials

$\alpha$ -MEM without  $\text{NaHCO}_3$  (Gibco #11900, Invitrogen), buffered with 25ml/L 1M HEPES, then supplemented as for complete medium, for 3D culture 4.44ml glucose was added as for HG medium (supplements Table 2.1 , page 45)

$^{14}\text{C}$ - $\text{NaHCO}_3$  2 $\mu\text{Ci}/\mu\text{l}$  (GE Healthcare #CFA3)

$^{14}\text{C}$ -Arginine, L-Guanido 1 $\mu\text{Ci}/\mu\text{l}$  (Perkin Elmer#NEC453)

$^{14}\text{C}$ -Urea  $1\mu\text{Ci}/\mu\text{l}$  (GE Healthcare #CFA41)

Jack Bean Urease Type III

Acetone (BDH)

Butanol (BDH)

Glacial Acetic Acid (BDH)

Scintillant InstaGel Plus (Packard Bioscience)

Flexible Cellulose Acetate-backed TLC plates (Merck)

Glass TLC tank

Beckman Scintillation Counter LS6500 (this model is factory set to compensate for quenching and counting efficiency and automatically calculates DPM from CPM)

Edwards Freeze Dryer

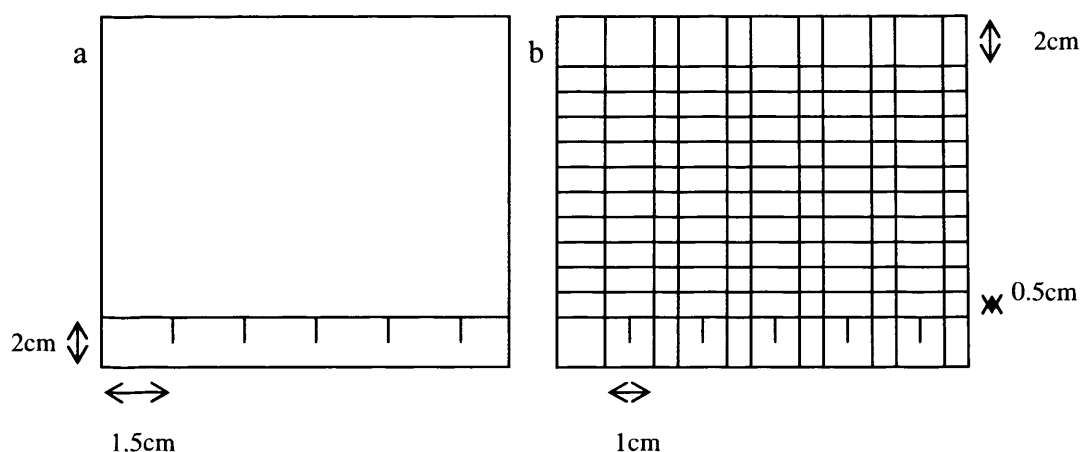
Water bath

*Method:*

Experiments with  $^{14}\text{C}$ - $\text{NaHCO}_3$  were conducted in HepG2 and C3A monolayer and 3-D culture, and in monolayer primary human hepatocyte culture, all plated as previous. When cells reached 50-60% confluency, the medium was changed to supplemented  $\alpha$ -MEM without  $\text{NaHCO}_3$  buffered with HEPES. Cells were spiked with  $25\mu\text{l}$  ( $50\mu\text{Ci}$ ) in  $2\text{ml}$   $^{14}\text{C}$ - $\text{NaHCO}_3$  and the plates sealed with plastic film to prevent the loss of  $^{14}\text{C}$ -labelled  $\text{CO}_2$ . Plates were returned to the incubator ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ) for 24 hours after which time the medium was collected and the cells harvested by scraping, pelleted and washed with cold  $1\times\text{PBS}$  twice. 3D culture HepG2 cells were prepared with  $0.5\times 10^6$  cells/ml alginate and  $0.25\text{ml}$  alginate beads/ $8\text{mls}$  medium, medium was changed on day 8 (approximately  $1\times 10^6$  cells) and spiked with  $37.5\mu\text{l}$  ( $75\mu\text{Ci}$ ) in  $3\text{ml}$   $^{14}\text{C}$   $\text{NaHCO}_3$ , plates sealed and incubated. Medium and cells were harvested from the alginate as described in section 2.1.2.1, page 48. Primary human hepatocytes were plated onto collagen at  $2\times 10^6$  cells per well in a six well plate. The next day the medium was changed and  $25\mu\text{l}$  ( $50\mu\text{Ci}$ )/ $2\text{ml}$   $^{14}\text{C}$ - $\text{NaHCO}_3$  added, as for HepG2 and C3A cells. The hepatocytes were harvested as for the monolayer cultures. The medium and cell lysates were frozen at  $-80^\circ\text{C}$ , lyophilised and then resuspended in  $40\mu\text{l}$  of cold water. Duplicates of the medium samples were urease treated to confirm the presence of urea by adding  $200\text{U}$  urease (stock urease  $4000\text{U}/\text{ml}$  in  $1\times\text{PBS}$ ) per sample and incubating at  $25^\circ\text{C}$  for 20 minutes.

The TLC plate was set up as in Figure 3.5a (page 66) and  $10\mu\text{l}$  of each sample was spotted onto the cellulose plate  $1\mu\text{l}$  at a time, at  $1.5\text{cm}$  intervals, drying the spots between each addition. The solvent was prepared fresh using butanol, acetone, water and glacial

acetic acid in the ratio of 35:35:20:10. The bottom 1cm of the plates were immersed into 200ml of solvent, and run until the front was 2cm from the top of the plate. The plates were air dried and marked into 1cm strips along the length of the plate for each sample run (Figure 3.5b). Each strip was then divided into 0.5cm wide sections which were cut, sequentially, from the bottom to the top of the plate into scintillation vials, 2ml scintillation fluid added, the vials vortexed briefly and the counts, recorded as DPM, taken for 10 minutes on a Beckman scintillation counter. Counts were corrected for protein (section 2.1.5, page 50).



*Figure 3.5 a and b: Set up of TLC plates for radiolabelled urea detection; (a) before running the samples, and (b) after running the samples marked with pencil in 1cm x 0.5cm strips.*

#### 3.1.1.4 TLC and Ninhydrin detection of $^{14}\text{C}$ -Labelled Urea Cycle Intermediates

To establish the functionality of the urea cycle, the incorporation of  $^{14}\text{C}$ - $\text{NaHCO}_3$  into the cycle to produce  $^{14}\text{C}$ -labelled urea cycle amino acids, specifically 1- $^{14}\text{C}$ -arginine and 1- $^{14}\text{C}$ -citrulline, was determined. TLC was used to separate the amino acids and they were detected on the plate with ninhydrin, which causes a colour change making individual spots visible. In the following experiment, the cell lysates from the above experiment were used.  $^{14}\text{C}$ -label incorporation in each spot was then determined by excising the spot into scintillation fluid and scintillation counting.

##### *Materials:*

1mg/ml Citrulline in water  
 1mg/ml Arginine in water  
 1mg/ml Ornithine in water  
 Acetonitrile (BDH)  
 Formic acid 88% (BDH)

Ninhydrin 0.2% in ethanol

Scintillant InstaGel Plus

Glass-backed silica-coated TLC plates (Merck)

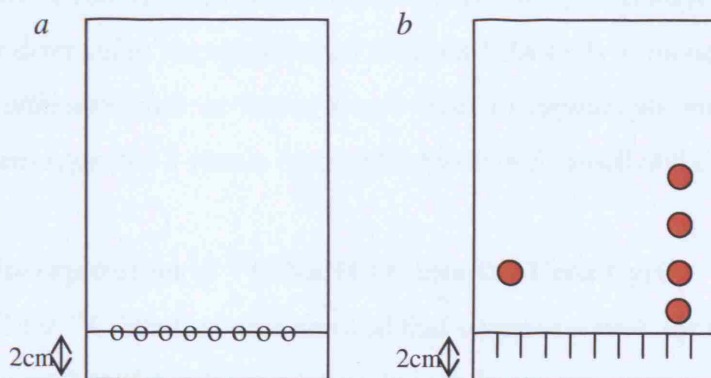
Glass TLC tank

Beckman Scintillation Counter LS6500

*Method:*

Initially, unlabelled standards were prepared, 1mg/ml of citrulline, arginine and ornithine and 5 $\mu$ l run on a TLC plate to obtain r.f. values. Thereafter a 1:1:1 mix of these amino acids was used and 10 $\mu$ l were spotted onto the TLC plate (Figure 3.6a). The cell lysates collected from the  $^{14}\text{C}$ -NaHCO<sub>3</sub> labelling experiment (3.1.1.2) were used, with 10 $\mu$ l of the lyophilised and resuspended lysates spotted onto the silica-coated TLC plate, 1 $\mu$ l at a time, drying the spot between each addition. The solvent was prepared fresh using acetonitrile, formic acid 88% and water in the ratio of 80:10:20. The bottom 1cm of the plates were immersed into 220ml of the solvent and run until the front was 2cm from the top of the plate.

The plates were air dried, sprayed generously with ninhydrin solution and placed in an oven preheated to 110°C. The plates were removed once the reddish-brown colouration of the amino acid-ninhydrin complex had appeared and then allowed to cool (Figure 3.6b). The individual spots were excised using a scalpel into a scintillation vial to which 3ml of scintillation fluid was added and the mixture vortexed. The  $^{14}\text{C}$  was recorded as DPM and measured for 10 minutes on a scintillation counter, and counts corrected for protein (section 2.1.5, page 50).



*Figure 3.6 a and b: Set up of glass-backed plates for radiolabelled amino acid detection. 3.6a before running the TLC plate and b after running the TLC plate and treating with ninhydrin.*

### 3.1.2 Results

#### 3.1.2.1 Urea Nitrogen Assay

This method proved too insensitive to use for urea detection. Whilst a good standard curve could be generated, urea in conditioned medium samples from HepG2, C3A cells and primary human hepatocytes could barely be detected above background levels.

#### 3.1.2.2 GC-MS of Urea

GC-MS showed that urea could be detected in HepG2 and C3A conditioned medium, with HepG2 cells producing significantly less than C3A cells, although urea from hepatocyte samples was ~7-fold greater (Figure 3.7).

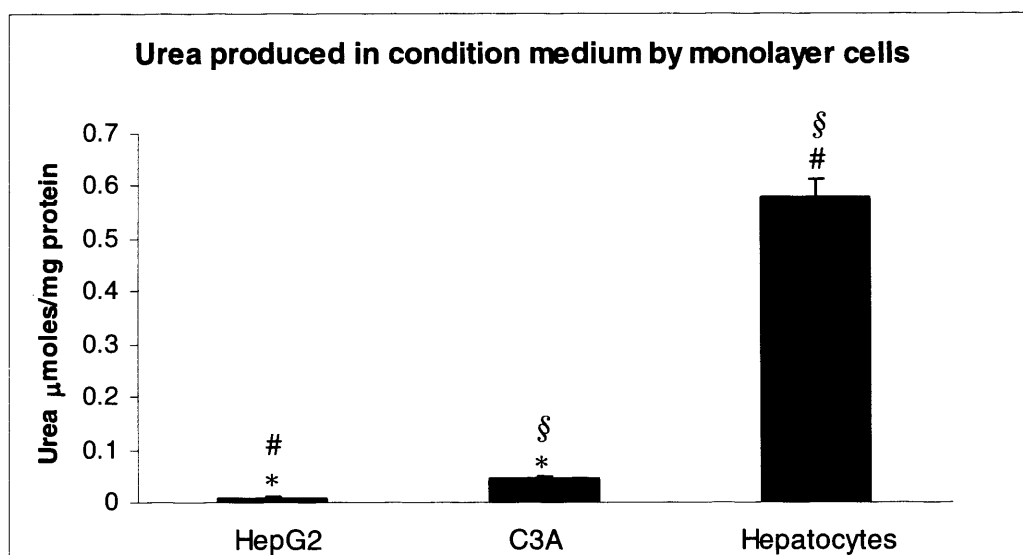


Figure 3.7: Urea detected in conditioned medium of monolayer cells by GC-MS. Urea could be detected in the conditioned medium C3A cells in monolayer culture and less in HepG2 although both at lower levels than in hepatocyte medium, ( $n=3$ , mean $\pm$ SD, experiment repeated 3 times, \* $p<0.005$ , # $p<0.005$ , \$ $p<0.005$ ).

#### 3.1.2.3 Incorporation of $^{14}\text{C}$ - $\text{NaHCO}_3$ into the Urea Cycle

The TLC for  $^{14}\text{C}$ -labelled urea showed that whereas a peak for urea could be detected in conditioned medium from primary human hepatocytes, urea was not detected in medium collected from monolayer or 3D-cultured HepG2 or C3A cells (Figure 3.8, page 69). Urease treatment of the samples confirmed this peak as urea, seen by the removal of the urea peak in hepatocyte samples.



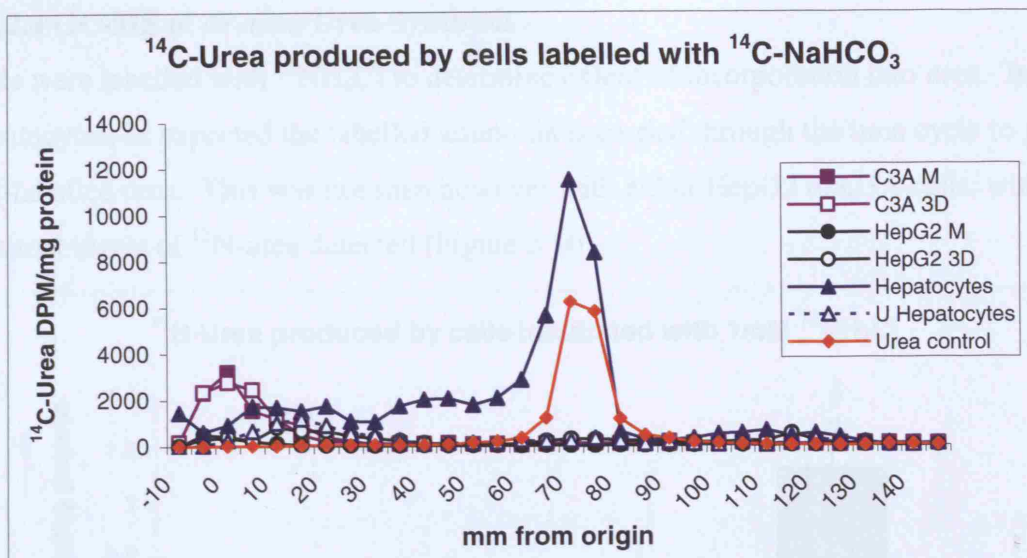


Figure 3.8:  $^{14}\text{C}$ -urea production. A peak for  $^{14}\text{C}$ -urea is detected in hepatocyte conditioned medium but not in HepG2 or C3A medium. The urea peak for the urea control (red diamond) overlaps with that of hepatocytes (solid blue triangle). Urease specifically removes this peak in treated samples (U hepatocytes-open triangle dashed blue line), ( $n=2$ , repeated twice).

Results for the  $^{14}\text{C}$ -labelled amino acids showed that whilst labelled citrulline and arginine were detected in hepatocyte samples, they were not found in either HepG2 or C3A cell lysates (Figure 3.9).

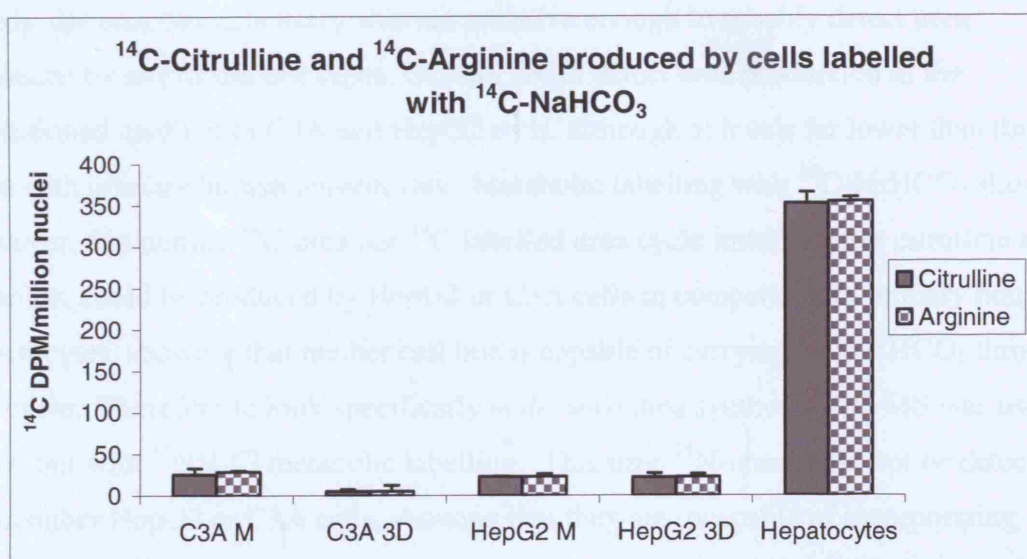


Figure 3.9:  $^{14}\text{C}$ -labelled amino acids production. Citrulline (solid bar) and arginine (chequered bar) are detected in hepatocyte, but not in HepG2 or C3A cell lysates, ( $n=3$ , mean $\pm$ SD, repeated twice).



### 3.1.2.4 GC-MS of *de novo* Urea Synthesis

Cells were labelled with  $^{15}\text{NH}_4\text{Cl}$  to determine extent of incorporation into urea. In hepatocytes, as expected the labelled ammonia is carried through the urea cycle to give  $^{15}\text{N}$ -labelled urea. This was not seen however with either HepG2 or C3A cells, with minimal levels of  $^{15}\text{N}$ -urea detected (Figure 3.10).

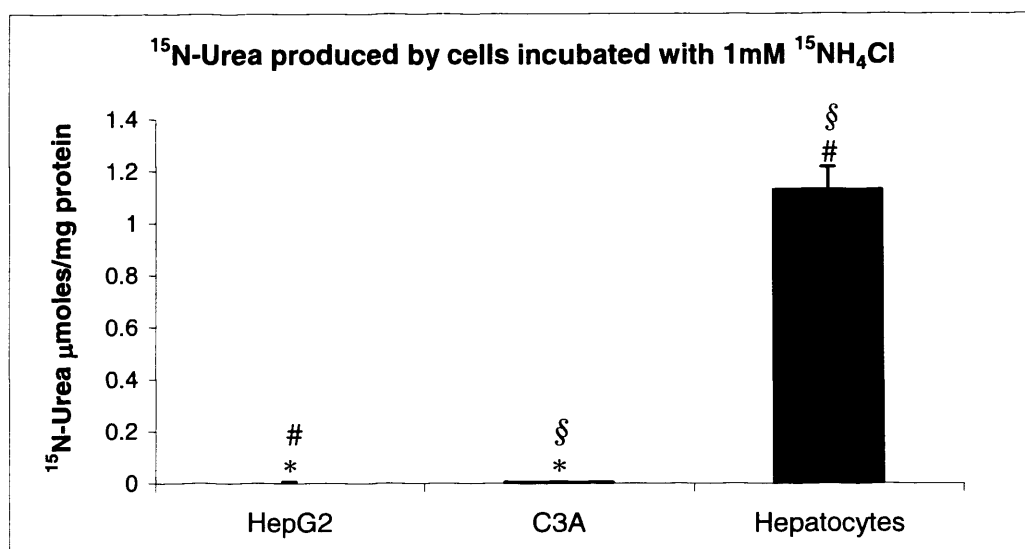


Figure 3.10:  $^{15}\text{N}$ - urea produced by monolayer cells. Labelled urea could be not be detected in conditioned medium of either HepG2 or C3A but was readily incorporated into by hepatocytes, ( $n=3$ ,  $\text{mean} \pm \text{SD}$ , repeated twice,  $*p=n.s.$ ,  $\#p<0.005$ ,  $\$<,0.005$ ).

### 3.1.3 Discussion

Whilst the urea nitrogen assay was not sensitive enough to reliably detect urea produced by any of the cell types, GC-MS could detect urea production in the conditioned medium of C3A and HepG2 cells, although at levels far lower than those seen with primary human hepatocytes. Metabolic labelling with  $^{14}\text{C}$ - $\text{NaHCO}_3$  showed, however, that neither  $^{14}\text{C}$ -urea nor  $^{14}\text{C}$ -labelled urea cycle intermediates citrulline and arginine, could be produced by HepG2 or C3A cells in comparison to primary human hepatocytes, showing that neither cell line is capable of carrying  $^{14}\text{C}$ - $\text{NaHCO}_3$  through the cycle. Therefore to look specifically at *de novo* urea synthesis, GC-MS was used again but with  $^{15}\text{NH}_4\text{Cl}$  metabolic labelling. This time  $^{15}\text{N}$ -urea could not be detected from either HepG2 or C3A cells, showing that they are incapable of incorporating  $^{15}\text{NH}_4\text{Cl}$  into  $^{15}\text{N}$ -urea, whereas this was readily done by primary human hepatocytes.

It was expected that HepG2 cells would neither synthesize urea nor convert  $^{15}\text{N}$ -ammonia to  $^{15}\text{N}$ -urea, but it was surprising that C3A cells do synthesize urea but do not

produce labelled-urea via the urea cycle. The observation that C3A cells produced significantly more urea than HepG2 cells is consistent with the literature, where C3A cells are cited as producing urea, as a function of the urea cycle. However, metabolic labelling data does not support these findings. One possible reason for this could be that the source of urea production is via a urea cycle-independent mechanism, which may not be involved with ammonia detoxification; this hypothesis is further discussed and explored in Chapter 5.

Therefore, these results clearly indicate that ammonia cannot be detoxified via the urea cycle in either HepG2 or C3A cells. The reasons for why urea production is decreased are investigated in the following experiments.

## **3.2 Quantification of Gene Expression in HepG2 and C3A Cells Using Real Time RT-PCR**

Real Time RT-PCR analysis of the five urea cycle genes, CPSI, OTC, AS, AL and Arginase I, was carried out in order to determine whether the urea cycle genes are expressed at the mRNA level. Standard curves for each gene were constructed then expression levels were expressed relative to 18S rRNA levels.

### **3.2.1 Methods**

#### *Materials*

HepG2, C3A, Primary Human Hepatocytes and Liver cDNA

Oligonucleotides (10 $\mu$ M working stock) (designed using VectorNTI version 10 software)

#### *Method*

Firstly, primers were designed using Vector NTI software to amplify gene specific fragments of 100-160bp (Table 3.1, page 72). These were BLAST searched to ensure they were gene specific. Standard curves were generated for each target gene and used to quantify gene expression, as described in Chapter 2 (section 2.2.5, page 54). Real Time RT-PCR was carried out on HepG2 and C3A cells, for both monolayer and 3D culture, primary human hepatocytes and human liver cDNA using each set of primers.

Table 3.1: The sequences listed for real time RT-PCR primers in the 5' to 3' orientation.

Sequence	Primer forward	Primer reverse	Fragment bp
CPS I	GGCCATCCATCCTCTGTTGC	GCTAAGTCCCAGTTCATCCA	148
OTC	ATCTGAGGATCCTGTAAACAATG	CCTTCAGCTGCACTTTATTTGTAG	150
AS	TCGTGCATCCTCGTGTGGCTGAA	CCACAAACTCCCTGCTGACATCC	135
AL	GAGGTGCGGAAGCGGATCAATGTC	TTGGTGCAGTAGAGGATGAGGTCC	145
Arginase I	GCAGAAGTCAAGAAGAACGG	GGTTGTCAGTGGAGTGTG	139
18S rRNA	CTTAGAGGGACAAGTGGCG	GGACATCTAAGGGCATCACA	70

To make cDNA for HepG2 and C3A monolayer cells and primary human hepatocytes, cells were plated in wells as previously described and harvested when confluent or on day 2, respectively, by washing cells 3x with sterile ice-cold 1xPBS then adding denaturing reagent from the RNAgents kit. HepG2 and C3A cells 3D culture were harvested on day 8, and the cell pellet resuspended in denaturing reagent. Sections of human liver (from liver resection) were snap frozen in liquid nitrogen, crushed using a pestle a mortar and resuspended in denaturing reagent from the RNAgents kit. RNA was isolated from all these samples and cDNA made as described in Chapter 2.0. In figure legends, n=6 denotes 6 different liver samples, 6 hepatocyte preparations from different livers (although not from matching livers), and 6 separate wells of HepG2 and C3A cells. The real time PCR conditions for each gene are in Table 3.2.

Table 3.2: Real time RT-PCR cycling conditions for the various genes.

Target	Hold 95°C	Denature 94°C	Anneal	Elongate 72°C	Hold 60°C	Cycle No
CPS I	15min	30sec	59°C 30sec	40sec	30sec	x40
OTC			59°C 30sec			x45
AS			57°C 30sec			x40
AL			57°C 30sec			x40
ArgI			58°C 30sec			x45
18S			60°C 30sec			x30

### 3.2.2 Results

Real Time RT-PCR results showed that levels of all urea cycle genes were consistently higher in the whole human liver than in any of the other cell preparations including those of primary human hepatocytes. In both HepG2 and C3A cells there was an almost complete absence of OTC and arginase I expression in both monolayer and 3D-cultured cells, as seen in Figures 3.11 and 3.12, respectively (page 73).

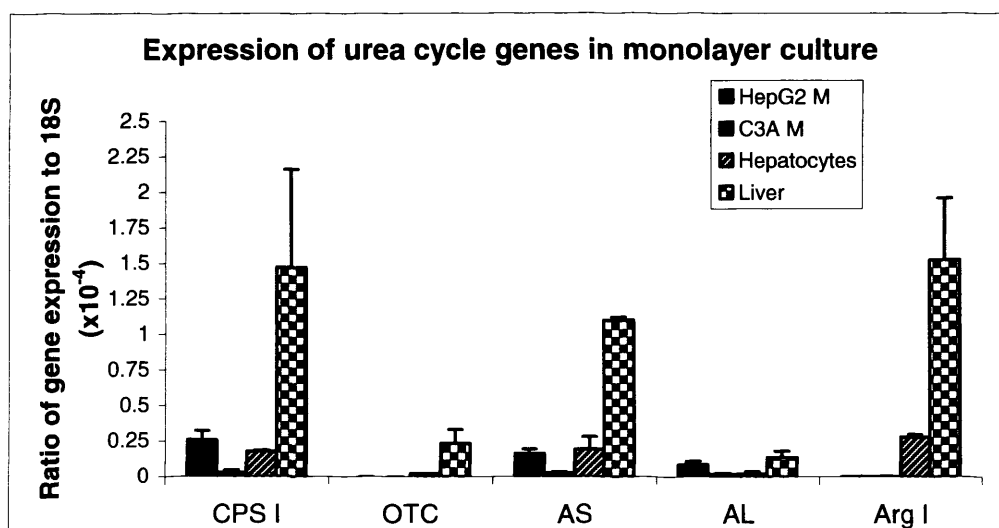


Figure 3.11: The expression of the five urea cycle genes in monolayer cells. CPSI; OTC; AS; AL and arginase I in HepG2 and C3A cells in comparison to primary human hepatocytes and human liver samples, (n=6, mean±SD, repeated 3 times). Gene expression levels were significantly different between liver and HepG2 and C3A cells for all genes except AL.

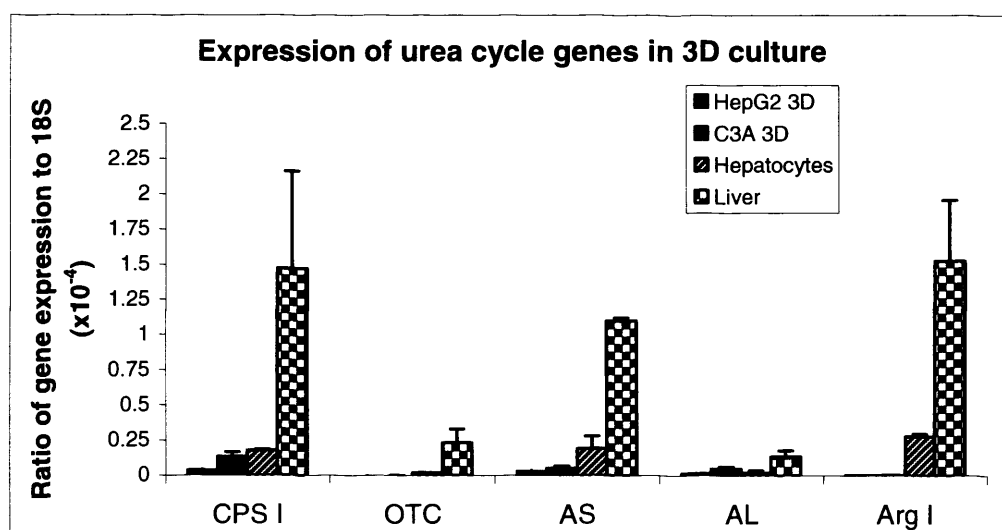


Figure 3.12: The expression of the five urea cycle genes in 3D culture. CPSI; OTC; AS; AL and arginase I in HepG2 and C3A 3D-culture in comparison to primary human hepatocytes and human liver samples, (n=6, mean±SD, repeated 3 times). Gene expression levels were significantly different between liver and HepG2 and C3A cells for all genes.

### 3.2.3 Discussion

The data obtained from the Real Time RT-PCR analysis clearly show that the lack of urea and urea cycle intermediate synthesis, seen in previous experiments, is due to the

complete absence of OTC and arginase I mRNA expression. The three other urea cycle genes were expressed although at lower levels than in primary human hepatocytes or the liver. This may be indicative of the general down-regulation of the urea cycle or, for example with AL, where expression is consistently low in all samples, the gene may possibly be in low abundance or the transcript is rapidly degraded after synthesis. However, both AS and AL, and possibly CPSI, have other roles to play in the cell, and expression levels seen may be consistent with these roles. For example, AS and AL are also involved in nitric oxide (NO) production as part of the NO cycle and as seen in (91), when these enzymes are expressed as part of this cycle, their overall expression is far lower than in cells where they are expressed as part of the urea cycle. In addition, CPSI may have some functional overlap with CPSII, in that it may regulate the amount and availability of carbamoyl phosphate which CPSII would incorporate into pyrimidine biosynthesis (92).

To determine whether the genes expressed are fully functional, enzyme assays were carried out as described in the following experiments.

### **3.3 Determining Functional Activity of the Urea Cycle Enzymes in HepG2 and C3A Cells**

Enzyme assays were carried out to determine whether CPSI, AS and AL were functional, and to confirm the absence of OTC and arginase I in HepG2 and C3A cells:

- Assay 1) The activities of CPSI and OTC were investigated by labelling cell lysates with  $^{14}\text{C}$ - $\text{NaHCO}_3$  and  $^3\text{H}$ -ornithine, to give  $1\text{-}^{14}\text{C}^3\text{H}$ -citrulline, (Figure 3.13, page 75). This assay was performed with and without the addition of OTC enzyme to determine whether the addition of exogenous OTC could replace lost endogenous OTC expression in HepG2 and C3A cells to give  $^{14}\text{C}^3\text{H}$ -citrulline, thereby indicating whether CPSI is functional.
- Assay 2) AS and AL activities were determined by labelling cell lysates with  $1\text{-}^{14}\text{C}$ -citrulline to give  $1\text{-}^{14}\text{C}$ -arginine (Figure 3.13, page 75). For assays 1 and 2, the amino acids were dansylated, separated by TLC, the spots visualised with UV, excised and scintillation counted. Dansyl chloride binds to the amine group and fluoresces on UV exposure and is therefore more sensitive than ninhydrin.

- Assay 3) Arginase activity was determined by incubating cell lysates with  $^{14}\text{C}$ -arginine to give  $^{14}\text{C}$ -urea and TLC performed as previously described.

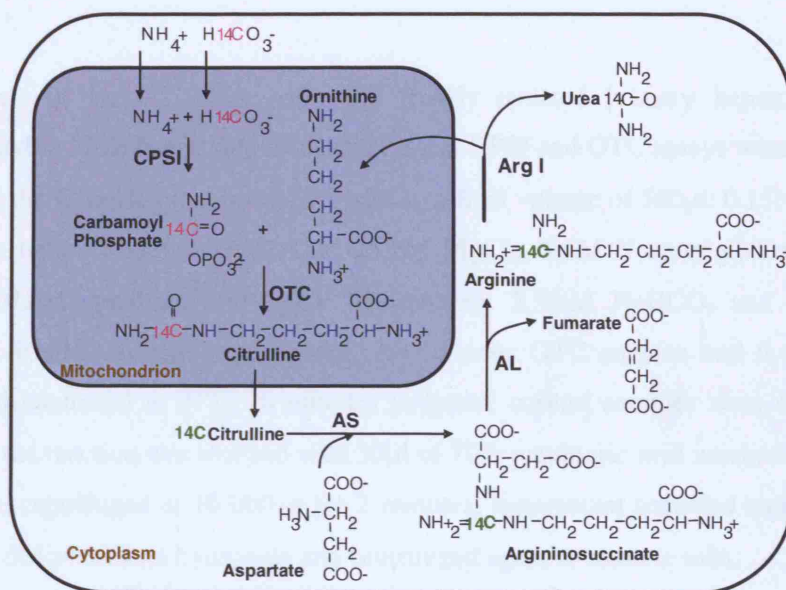


Figure 3.13: Labelling methods of the enzyme assays.  $^{14}\text{C}$ - $\text{NaHCO}_3$  (pink) and  $^3\text{H}$ -ornithine (blue) are incorporated into citrulline.  $^{14}\text{C}$ -citrulline (green) is incorporated into arginine.

### 3.3.1 Methods

Enzyme assays were from Wu *et al* (93) with modifications described in (94)

#### 3.3.1.1 CPSI and OTC: Labelling with $^{14}\text{C}$ - $\text{NaHCO}_3$ and $^3\text{H}$ -labelled Ornithine

##### Materials

KHB buffer (118mM NaCl, 4.8mM KCl, 1.2M  $\text{KH}_2\text{PO}_4$ , 2.1M  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 2mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 25mM HEPES, pH 7.2, and 1mM DTT, 0.5% Triton-X 100 added)

0.15M Potassium phosphate buffer, pH7.5

25mM ATP

25mM  $\text{MgCl}_2$

5mM N-acetylglutamate

10mM  $\text{NH}_4\text{Cl}$

10mM Ornithine

5 $\mu\text{Ci}$   $^3\text{H}$ -ornithine

2.5mM  $\text{NaHCO}_3$

5 $\mu\text{Ci}$   $^{14}\text{C}$ -  $\text{NaHCO}_3$

10mM Carbamoyl phosphate

10 units OTC enzyme

70% Perchloric acid

5M Potassium hydroxide

Dansyl chloride 5mg/ml in acetone

### *Method*

Cell pellets of HepG2, C3A cells and freshly isolated primary hepatocytes were resuspended in KHB buffer and sonicated on ice. CPSI and OTC assays were carried out by adding the following to a microfuge tube to a final volume of 500µl: 0.15M potassium phosphate buffer pH7.5, 25mM ATP, 25mM MgCl<sub>2</sub>, 5mM N-acetylglutamate, 10mM NH<sub>4</sub>Cl, 10mM ornithine and 5µCi <sup>3</sup>H-ornithine, 2.5mM NaHCO<sub>3</sub> and 5µCi <sup>14</sup>C-NaHCO<sub>3</sub>, 10mM carbamoyl phosphate, +/- 10 units OTC enzyme and 0.4mg protein added and incubated at 37°C 20 minutes (negative control samples were stopped at 0 minutes), the reaction was stopped with 30µl of 70% perchloric acid incubated on ice for 5 minutes, centrifuged at 10 000xg for 2 minutes, supernatant removed and neutralised with 80µl 5M potassium hydroxide and centrifuged again to remove salts.

#### **3.3.1.2 AS and AL: Labelling with <sup>14</sup>C-labelled Citrulline**

For AS and AL the following were added to a microfuge to a final volume of 200µL: 75mM potassium phosphate buffer pH7.5, 5mM citrulline and 5µCi <sup>14</sup>C-citrulline, 10mM aspartate, 5mM ATP and 0.4mg protein added, incubated at 37°C 20 minutes (negative control samples were stopped at 0 minutes), then 5mM argininosuccinate and 65mM EDTA were added and incubated for a further 15 minutes. The reaction was stopped and supernatant neutralised with 12µl perchloric acid and 32µl KOH, and as above the samples were centrifuged.

To dansylate the supernatants, the protocol of Weiner *et al.* (95) was followed with modification. Briefly, 5mg/ml dansyl chloride in acetone was prepared and 250µl and 100µl added to the CPSI OTC and AS AL supernatants, respectively. These were incubated at 50°C for 40 minutes, the reaction was stopped with 25µl and 10µl 88% formic acid respectively. Amino acid standards were prepared by drying down 2.5mM each of arginine, ornithine, and citrulline in 0.01M HCl, resuspending in 0.5ml 0.1M NaHCO<sub>3</sub> and dansylating with 250µl dansyl chloride as above. The solvent was prepared fresh using ethylacetate: glacial acetic acid: methanol at 20:3:1. 10µl of the dansylated supernatant/standard mix was spotted onto glass-backed silica TLC plates and run for 2hours. The spots were visualized after 1.5 hours with long wave UV light. The spots corresponding to the citrulline or arginine standard were then excised into 3ml scintillation fluid, vortexed, and counted as previously (DPM) for <sup>14</sup>C and then counted for <sup>3</sup>H.

### **3.3.1.3 Arginase I: Labelling with $^{14}\text{C}$ -labelled Arginine**

The urea was separated and detected using TLC as in section 3.1.1.3 (page 64).

#### *Materials*

0.25mM manganese sulphate/75mM glycine buffer, pH9.7

100mM arginine

$^{14}\text{C}$ -Arginine, L-Guanido 1 $\mu\text{Ci}/\mu\text{l}$

#### *Method*

Cell lysates were prepared in KHB buffer as described above. The following were combined: 90 $\mu\text{l}$  buffer, 2mM final arginine, 5 $\mu\text{l}$   $^{14}\text{C}$ -arginine and 0.4mg protein. Samples were incubated at 37°C for 20 minutes, the reaction stopped with TCA (4% final concentration) and centrifuged at 10000xg to remove debris. 10 $\mu\text{l}$  of the sample was spotted onto a TLC plate and the method followed as for Urea TLC in section 3.1.1.3. The assay had previously been optimised for the ratio of labelled to unlabelled arginine by using a constant amount of labelled arginine (5 $\mu\text{l}$ ) and an increasing concentration of unlabelled arginine (0, 2, 4, 6, 8 mM).

## **3.3.2 Results**

### **3.3.2.1 CPSI and OTC Activity**

To confirm mRNA expression data for CPSI, OTC, AS, AL and arginase I, radiolabelled enzyme assays were conducted. CPSI activity was determined indirectly by detecting the incorporation of  $^{14}\text{C}$ - $\text{NaHCO}_3$  and  $^3\text{H}$ -ornithine into dual-labelled 1- $^{14}\text{C}^3\text{H}$ -citrulline. It is expected that dual-labelled citrulline can only be produced in the presence of both functional CPSI and OTC. This is illustrated by primary human hepatocytes with the production of dual-radiolabelled citrulline. HepG2 and C3A cells however are incapable of producing 1- $^{14}\text{C}^3\text{H}$ -citrulline, however it can be made when exogenous OTC is added to the cell lysates, (Figures 3.14, page 78).



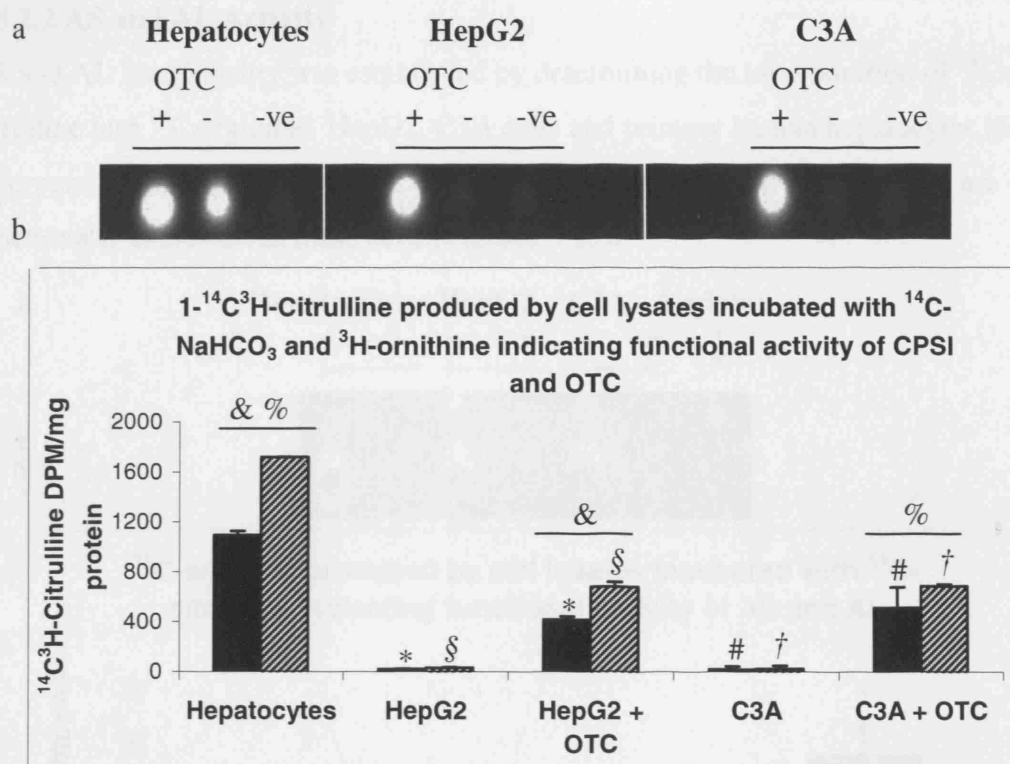


Figure 3.14 a and b: Functional activity of CPSI and OTC. Figure a) dansylated <sup>14</sup>C<sup>3</sup>H-citrulline spots in samples for hepatocyte positive control, HepG2s and C3A cells. Cell lysates were incubated either with (+) or without (-) OTC, and negative controls (-ve) were samples stopped at 0 minutes. Figure b shows the quantitation (DPM) for radiolabelled citrulline production, <sup>14</sup>C (solid bar) and <sup>3</sup>H (diagonal bar) from primary human hepatocytes, HepG2 and C3A cells with (+) and without added OTC enzyme, (n=3, mean±SD, <sup>14</sup>C data: \*p<0.0001, §p<0.0001, #p,0.05, †p<0.0001, &p<0.0001, %p<0.005), there were no significant differences between the HepG2 and C3A data either with or without OTC).

These results show that <sup>14</sup>C-NaHCO<sub>3</sub> and <sup>3</sup>H-ornithine were incorporated into <sup>14</sup>C<sup>3</sup>H-citrulline by HepG2 and C3A cells only in the presence of added OTC enzyme. This confirms that CPSI is functional in both HepG2 and C3A cells. These data also supports the mRNA results in that OTC is absent; this pathway can be restored with exogenously added OTC. In addition, to ensure that <sup>14</sup>C-citrulline could not be generated from <sup>14</sup>C-ornithine which could be produced via the incorporation of <sup>14</sup>C-NaHCO<sub>3</sub> through the Krebs cycle and into glutamate, cell lysates were incubated with <sup>14</sup>C-NaHCO<sub>3</sub> alone and the production of <sup>14</sup>C-ornithine measured. This showed that no <sup>14</sup>C-ornithine, hence no <sup>14</sup>C-citrulline, could be produced in this way.

### 3.3.2.2 AS and AL Activity

AS and AL functionality was established by determining the incorporation of  $^{14}\text{C}$ -citrulline into  $^{14}\text{C}$ -arginine. HepG2, C3A cells and primary human hepatocytes all incorporated  $^{14}\text{C}$ -citrulline into  $^{14}\text{C}$ -arginine, showing that these two enzymes are functionally expressed in these cells (Figures 3.15).

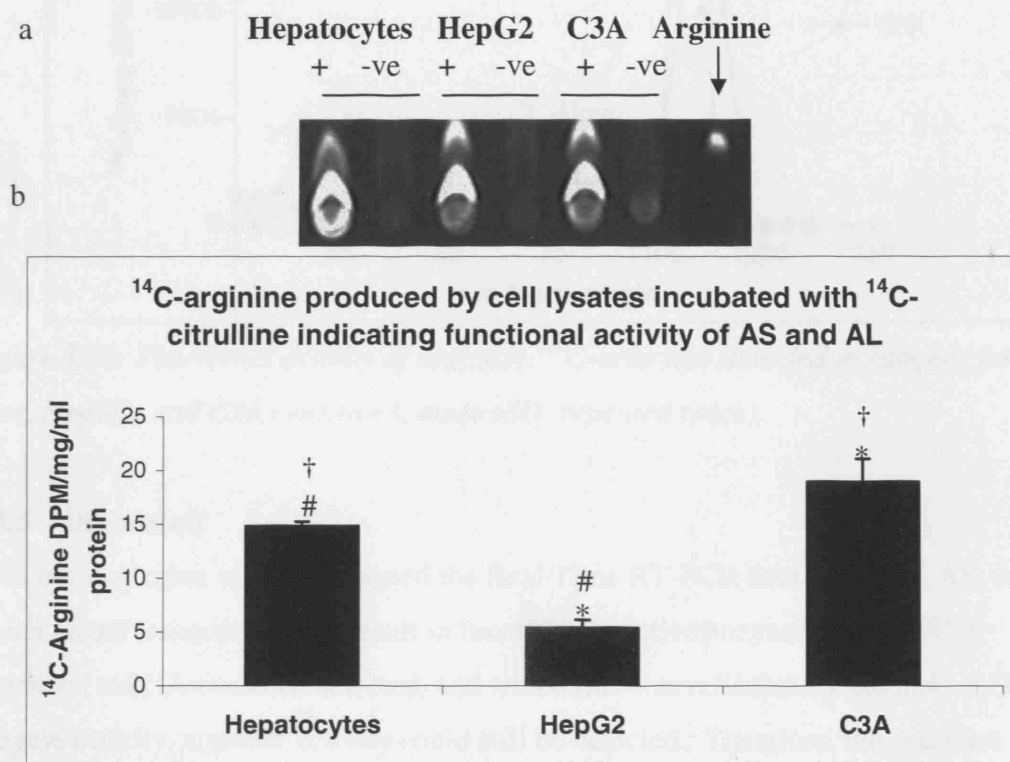


Figure 3.15 a and b: Functional activity of AS and AL. Figure a) dansylated  $^{14}\text{C}$ -arginine spots, in, in samples for hepatocytes, HepG2s and C3A cells (+), negative controls (-ve) were samples stopped at 0 minutes, and an arginine standard. Figure b) shows the quantitation (DPM) for radiolabelled arginine production from primary human hepatocytes, HepG2 and C3A cells, ( $n=3$ , mean $\pm$ SD, # $p<0.005$ , † $p=ns$ , \* $p<0.005$ ).

### 3.3.2.3 Arginase Activity

To determine whether the absence of arginase I mRNA was consistent at the protein level, arginase activity was assessed by determining the conversion of  $^{14}\text{C}$ -arginine to  $^{14}\text{C}$ -urea. Contrary to the Real Time RT-PCR results, arginase activity was detected in both HepG2 and C3A cells (Figure 3.16, page 80). As this was in contrast to the mRNA data, it was hypothesized that the labelled arginine could be converted to labelled urea by enzymes other than arginase I, which is only found as part of the urea cycle. This is explored further in chapter 4.

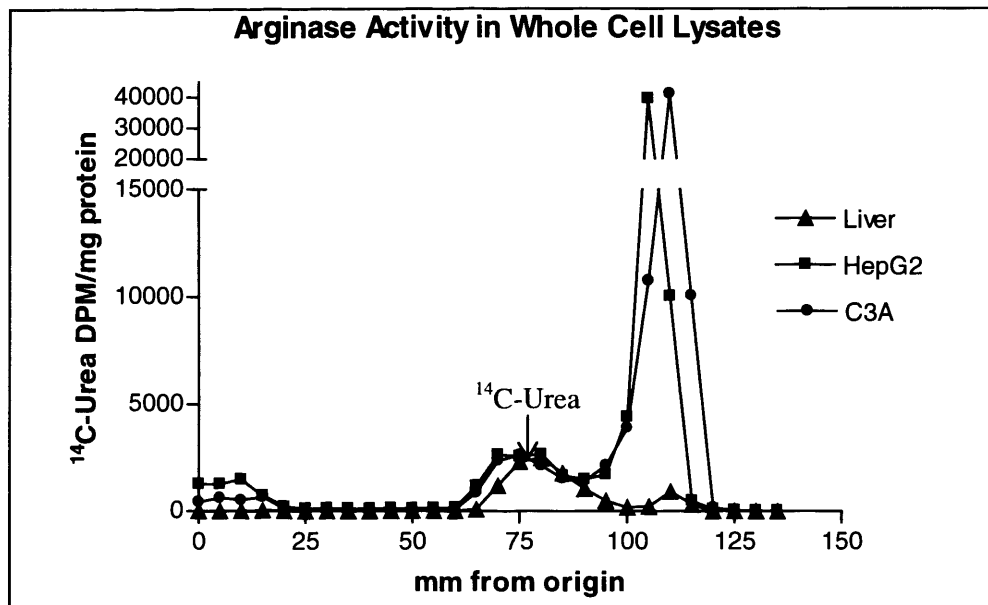


Figure 3.16: Functional activity of arginase.  $^{14}\text{C}$ -urea was detected in samples for liver, HepG2s and C3A cells, ( $n=3$ , mean $\pm$ SD, repeated twice).

### 3.3.3 Discussion

Urea cycle enzyme assays supported the Real Time RT-PCR data for CPSI, AS, AL which are all transcribed, and result in functionally active enzymes. No OTC or arginase I mRNA could be detected, and whilst this was reflected by the lack of OTC enzyme activity, arginase activity could still be detected. Therefore, the presence of arginase activity needed further investigation therefore, to determine whether arginine is being metabolised to urea by enzymes other than arginase I.

## 3.4 Conclusion

Human hepatocyte derived cell lines, such as HepG2 cells are one option for the biological component of the BAL. Ammonia detoxification is an essential function of the liver, and this, along with the synthesis of plasma proteins, must be mimicked by a BAL. Microarray array and functional studies have shown that HepG2 cells have up-regulated liver-like function in 3D culture, however urea production is minimal. The reasons therefore behind this down-regulation were examined at both a molecular and biochemical level. C3A cells, a cell line derived from subcloning HepG2 cells were also examined. These are reported to produce urea, where urea production is assumed to be reflective of ammonia detoxification via the urea cycle. Therefore, C3A cells could be considered to offer a suitable replacement to HepG2 cells as regards urea

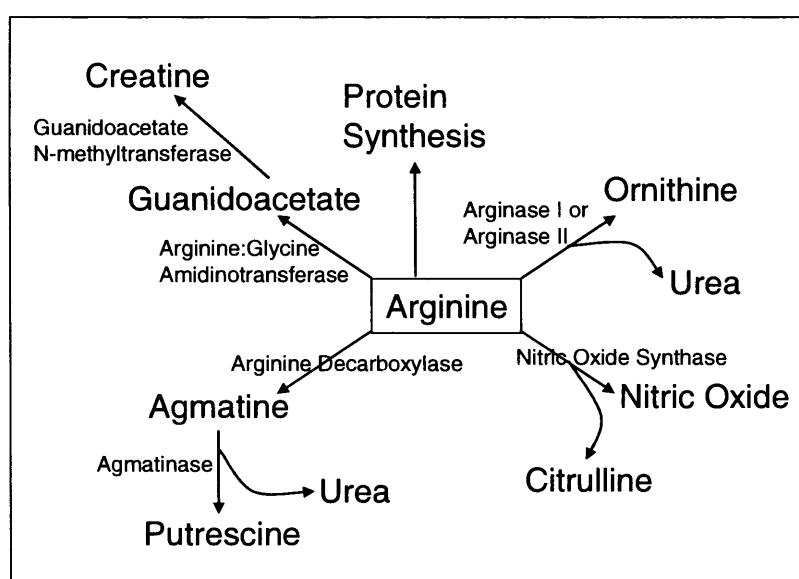
cycle function.

The data presented in Chapter 3 indicate that the urea cycle is non-functional in both HepG2 and C3A cells. The initial finding, showed that urea could be produced by these two cell lines, however upon further investigation, it was determined that ammonia could not specifically be removed by the urea cycle. These data was supported by using two different methods of metabolic labelling: i)  $^{14}\text{C}$ - $\text{NaHCO}_3$  and TLC, and ii)  $^{15}\text{NH}_4\text{Cl}$  and GC-MS. Both compounds are starting substrates for the urea cycle. These findings were explained by the lack of expression of two urea cycle genes, OTC and arginase I, in both HepG2 and C3A cells; although CPSI, AS and AL are expressed. Whilst this was mostly substantiated by the functional enzyme assays, contrary to the mRNA data, arginase activity was detected in cell lysates.

It was therefore postulated, that arginine could be metabolised by other enzymes to produce urea. Alternate mechanisms of urea production are explored in Chapter 4.

## Chapter 4: Alternative Mechanisms of Urea Production

A key function required by BAL systems is the provision of ammonia detoxification. As shown in Chapter 3, neither HepG2 nor C3A cells have a functional urea cycle, the pathway by which ammonia is removed in human hepatocytes. Interestingly however, C3A cells are cited in the literature as capable of producing urea and this has been assumed to be reflective of urea cycle function and hence ammonia detoxification by these cells. The findings presented have conclusively shown that neither HepG2 cells, or the HepG2 derived cell line C3A, can effectively detoxify ammonia into urea, and that urea cycle function is perturbed due to the absence of OTC and arginase I expression. However, despite the lack of arginase I mRNA expression, arginine could be converted to urea independently of arginase I enzyme activity. This led to the hypothesis that alternative mechanism of urea production from arginine metabolism were occurring independently of the urea cycle and ammonia detoxification. Figure 4.1 illustrates alternative metabolic pathways of arginine.



*Figure 4.1: Diagram of arginine metabolism. Arginine can be converted into a variety of products.*

This figure shows that urea can be produced as a by-product of arginine metabolism by arginase I, arginase II, and agmatinase via arginine decarboxylase (ADC). To investigate whether urea could be produced via alternative, urea cycle-independent, mechanisms, the following experiments were carried out:

- 1) Arginase II Activity: Since arginase II is located in the mitochondria, whereas arginase I is located in the cytosol, arginase assays were carried out on the two cell fractions.
- 2) Gene expression: Real Time RT-PCR was carried out to determine expression levels of arginase II, ADC and agmatinase.
- 3) Protein expression: Arginase I and II Western blots were used to determine the presence of the two proteins.
- 4) Inhibitor studies: To assess the degree of contribution to urea production by arginase II, inhibitor studies were conducted using N<sup>ω</sup>-Hydroxy-nor-L-Arginine (nor-NOHA) (96).

#### **4.1 Urea Production by HepG2 and C3A Cells**

The enzymes highlighted above are normally responsible for the following:

- i) Arginase II: this is an isoform of arginase I and also converts arginine to ornithine and urea. Importantly it is not normally found in the liver but is usually highly expressed in the kidney and prostate. Arginase II is a separate gene product located on a different chromosome, has a different cellular distribution in that it is mitochondrial, and the main purpose for arginine metabolism is for ornithine production as a precursor for polyamine, glutamate and proline biosynthesis, in addition to regulating arginine levels for nitric oxide (NO) production. The differences between arginase I and II are summarized in Table 4.1, for reviews see (78;97;98).
- ii) ADC: converts arginine to agmatine and is the step before agmatinase. It is believed to be involved in regulating polyamine production and cell proliferation (99).
- iii) Agmatinase: only recently shown to be expressed in mammalian cells, it is involved in putrescine production via the conversion of agmatine to N-carbamoyl putrescine, with urea made as a by product (100-102).

*Table 4.1: Differences between arginase I and arginase II (103-108).*

	<b>Arginase I</b>	<b>Arginase II</b>
Chromosomal Location	6q23	14q24.1-q24.3
Subcellular Localisation	Cytosol	Mitochondria
Tissue Distribution	Predominantly liver	Extra-hepatic-especially in kidney and prostate.
Role	Urea cycle enzyme	Nitric oxide regulation Polyamine metabolism Glutamate and proline synthesis

#### **4.1.1 Methods**

##### **4.1.1.1 Arginase II Functional Activity**

Arginase I and arginase II exist in different subcellular locations, and are found in the cytosol and mitochondria, respectively. Functional activity of arginase II was assessed by metabolic labelling experiments using these two fractions.

##### **4.1.1.1.1 Cell Fractionation**

The protocol to obtain mitochondrial and cytosolic fractions was followed as in (109).

##### *Materials*

Mitochondrial Isolation Buffer (0.5M Tris HCl pH7.5 (10ml), 1M sucrose (25ml), 50mM MgCl<sub>2</sub> (10ml), 50mM EDTA (10ml), 50mM EGTA (10ml), 30mM DTT (10μl)), 4°C.

0.1M phenylmethylsulphonylfluoride (PMSF) in DMSO stock

Ice cold PBS

150mM KCl ice cold

KHB buffer (as previously described)

Cell scrappers

Tissue Homogeniser

Centrifuge 4°C

Microcentrifuge 4°C

Ultra-Centrifuge 4°C

##### *Method*

Cell fractions were prepared from HepG2 and C3A cells, and whole human liver. To the mitochondrial isolation buffer, 1mM final concentration PMSF was added fresh. Cells were grown as previously described in Chapter 2. 3xT175 flasks of cells were rinsed 3x

with ice cold PBS and then cells were scraped into 9ml of the buffer and homogenised thoroughly. 3g human liver were homogenised thoroughly in 9ml buffer. These homogenates were then centrifuged at 1000xg for 5 minutes to remove nuclei and cell debris. The supernatants were then centrifuged at 20000xg for 15 minutes to pellet the mitochondria. The supernatant was collected and centrifuged at 150000xg for 3 hours to obtain a clear cytosol, which was aliquotted and kept at -80°C. The mitochondrial pellet was washed 5x with KCl buffer. This step was used in order to remove arginase I bound to the outer membrane of the mitochondria (110). The mitochondria were then resuspended in KHB buffer, aliquotted and kept at -80°C.

#### **4.1.1.1.2 Succinate Dehydrogenase Assay**

The succinate dehydrogenase (SDH) assay was used to determine whether the mitochondria were 'viable' and how much mitochondrial contamination there was in the cytosolic fraction (111).

##### *Materials*

SDH reagent (100mM Triethanolamine HCl pH8.3, 0.5mM EDTA, 2mM KCN or NaCN, 2mM Iodonitrotetrazolium Chloride, 12g/L Cremaphor EL, +/-20mM Succinate)

Water bath 30°C

Spectrophotometer at 500nm

Cuvettes

##### *Method*

The SDH reagent was made at the stated final concentrations either with or without succinate. The cytosolic protein was used as is, but the mitochondrial samples were sonicated on ice. To 990µl of buffer, 10µl of protein was added, incubated at 30°C for 6 minutes and a reading taken at 500nm. A standard curve was generated using liver homogenate. The assay was repeated without the addition of succinate to the buffer.

#### **4.1.1.1.3 Arginase II Assay**

The same arginase assay as in section 3.3.1.4 was used to assess arginase activity in the two cell fractions. Protein was quantified using the Bradford reagent as described in Chapter 2 (section 2.1.5, page 50).

#### **4.1.1.2 Arginase I and II Western Blot**

Western blots for arginase I and II were carried out. Arginase II was quantified by stripping the blot of arginase II and re-probing for β-actin. The bands were then



quantified using densitometry.

#### *Materials:*

Lysis buffer (50mM Tris-HCl, 150mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 0.5% sodium deoxycholate with 1mM PMSF)

#### *Loading Buffer*

Goat-anti-arginase I primary antibody (1:200) (Santa Cruz, #sc-18351)

Goat-anti-arginase II primary antibody (1:200) (Santa Cruz, #sc-18357)

Donkey anti-goat IgG secondary antibody (1:5000) (Santa Cruz #sc-2020)

4-12% Bis-Tris gels (Invitrogen)

1xMOPS running buffer (Invitrogen)

Bromphenol blue (5% in water)

Loading buffer (100mM Tris pH8.8, 60mM DTT, 40mM EDTA, 4% SDS, 25% glycerol)

Protein rainbow marker (GE Healthcare)

1xPBS

1xTBS (50mM Tris, 150mM NaCl, pH7.5)

Dried milk powder: 5% in 1xPBS, 5% in 1 xPBS plus 0.02% sodium azide, and  
5% in 1xTBS

Transfer buffer (43.3g Glycine (25mM), 9g Tris (192mM), 1.2g SDS, 300ml Methanol,  
to 3L, -20°C)

3MM paper (Whatman)

Sponge Pads

PVDF membrane (GE Healthcare)

X-ray Film (Kodak)

Enhanced Chemiluminescence Plus detection kit (GE Healthcare)

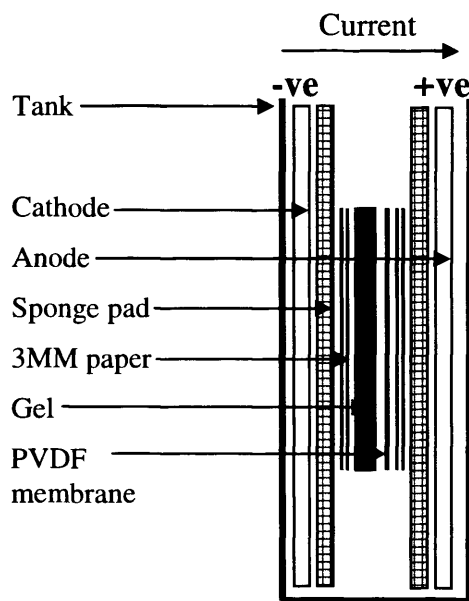
Western blot stripping reagent (Perbio)

Laser Densitometer

#### *Method*

C3A cells, HepG2 cells, rat kidney and human liver were homogenised in triple detergent lysis buffer with PMSF, and centrifuged to remove debris for 5 minutes at 16000xg. Protein was quantified using the BCA assay (Chapter 2, section 2.1.6, page 51). Protein was heated to 95°C for 5minutes with an equal volume of loading buffer. When cool, bromophenol blue was added. All the following steps were at room temperature unless otherwise stated. Equal amounts of protein (40µg) were loaded onto 12% gels. The protein was separated using SDS-PAGE electrophoresis in 1xMOPS running buffer, at 200 volts for 45 minutes. 10µl protein rainbow marker was used for reference. The gel was blotted onto Hybond P PVDF membrane in transfer buffer for 2 hours at 350mA

constant current at room temperature (Figure 4.2). The membrane was then blocked in 5% milk with PBS for 2 hours, then probed for arginase I with goat-anti-arginase I primary antibody (1:200 in 5% milk in PBS with azide) overnight at 4°C. The membrane was washed 3 x 15 minutes with PBS, then 1 x 15 minutes with TBS. A horse-radish peroxidase (HRP)-linked donkey anti-goat IgG secondary antibody was used for detection (1:5000 in 5% milk in TBS). After 1 hour at room temperature, the membrane was washed 4 x 15 minutes with TBS. Bands were visualized using Enhanced Chemiluminescence Plus (82).



*Figure 4.2: Transfer of protein from gel to PVDF membrane.*

The blot was then stripped using the stripping reagent according to manufacturers' protocol. Briefly, the membrane was washed 2x 20 minutes at room temperature, 1x 15 minutes PBS, and re-blocked with 5% milk in PBS plus azide, overnight. The membrane was probed for  $\beta$ -actin with goat-anti- $\beta$ -actin primary antibody (1:500) at room temperature for 2 hours, and then washed, probed with the secondary, and bands detected.

#### **4.1.1.3 Gene Expression Using Real Time RT-PCR**

Real Time RT-PCR was carried out as in Chapters 2 and 3 on HepG2 and C3A cells, for both monolayer and 3D culture, primary human hepatocytes and human liver cDNA using each set of primers, n=6 for all tissue types. Primers were designed and standard curves generated for each target gene as previously described. Primer sequences and PCR conditions are listed in Tables 4.2 and 4.3 (page 88).

Table 4.2: The sequences listed for real time RT-PCR primers in the 5' to 3' orientation.

Sequence	Primer forward 5'	Primer reverse 5'	Fragment bp
Arginase II	GCTGGCTTGATGAAAAGGCTCTCC	GCCATCTGACACAGCTCTGCTAAC	176
ADC	ATGGCTGGCTACCTGAGTGA	AAGGCAGCTACCTCGTCCGT	121
Agmatinase	CAGTGATGCTTGGGACAGTC	GCTGTCCTGAAGGTTGTAAAG	109
18S rRNA	CTTAGAGCGACAAGTGGCG	GGACATCTAAGGGCATCACA	150

Table 4.3: Real time PCR cycling conditions for the various genes.

Target	Hold 95°C	Denature 94°C	Anneal	Elongate 72°C	Hold 60°C	Cycle No
Arginase II	15min	30sec	59°C 30sec	40sec	30sec	x40
ADC			59°C 30sec			x45
Agmatinase			57°C 30sec			x40
18S rRNA			60°C 30sec			x30

#### 4.1.1.4 Inhibition of Arginase II Activity

Arginase II inhibition studies were carried out using N<sup>o</sup>-Hydroxy-nor-L-Arginine (nor-NOHA) (96). Arginase activity was determined by quantifying the conversion of <sup>15</sup>N<sub>2</sub>-guanido-arginine to <sup>15</sup>N-urea (112) and detected by GC-MS. This was done to assess the contribution to urea production by arginase II expression in HepG2 and C3A cells. The assay is a modification of the arginase assay described previously.

#### Materials

0.25mM manganese sulphate/75mM glycine buffer, pH9.7

KHB buffer

10mM nor-NOHA, diacetate salt (Calbiochem #399275)

100mM <sup>15</sup>N<sub>2</sub>-arginine

#### Method

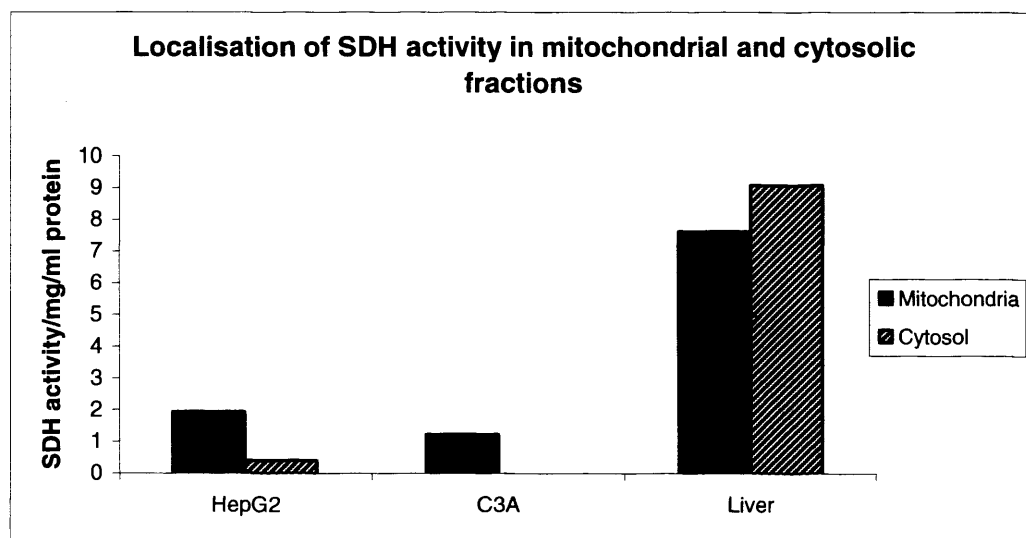
Cell lysates were prepared from HepG2 and C3A cells by freeze-thawing three times in 0.15M KHB buffer followed by centrifugation at 16000xg to remove debris. Kidney and liver samples were homogenized using a tissue homogeniser, then sonicated on ice and finally centrifuged. Protein was quantified using the Bradford reagent. The assays were carried out with modifications as follows: cell lysates were incubated with 0, 2, 20 and 200µM nor-NOHA in 0.25mM manganese sulphate/75mM glycine buffer, pH9.7 at 37°C for 5 minutes then 1mM <sup>15</sup>N-guanido-arginine (final concentration) added and samples

incubated at 37°C for a further 20 minutes. The reactions was stopped with TCA (5% final concentration) and centrifuged at 16000xg to remove debris. To quantify urea, the samples were purified, derivitized and analysed by GC-MS as previously described in section page. However, as  $^{15}\text{N}_2$ -urea is expected to be made during the arginase assay, 1mM unlabelled urea was used for the internal standard, instead of  $^{15}\text{N}_2$ -urea as used previously.

## 4.1.2 Results

### 4.1.2.1 Arginase II Functional Activity

Whilst the cell fractionation was successful for the HepG2 and C3A cells, the cytosolic fraction of the liver was highly contaminated with SDH. This was repeated and the same results obtained, thereby suggesting that the mitochondria were probably lysed or degraded and leaking enzymes into the cytosol (Figure 4.3). This may be because the livers had been subjected to chemotherapy in the patient and had been on ice for 2-4 hours in transit from donor to experiment.



*Figure 4.3: SDH activity in mitochondrial and cytosolic fractions. Whilst there was relatively little SDH contamination in the cytosol isolated from HepG2 and C3A cells, there was considerable SDH activity in the liver cytosol, (n=2, repeated twice).*

As seen in Figure 4.4 (page 90), there is arginase activity in both cytosolic and mitochondrial fractions of the liver, possibly indicating that if arginase I is in some way bound to the outer mitochondrial membrane, the KCl wash did not work efficiently. Despite this, the data for the arginase assay for HepG2 and C3A cells indicated that there was no arginase activity in the cytosol but there was

significant arginase activity in the mitochondrial fractions (Figure 4.5, page 90).

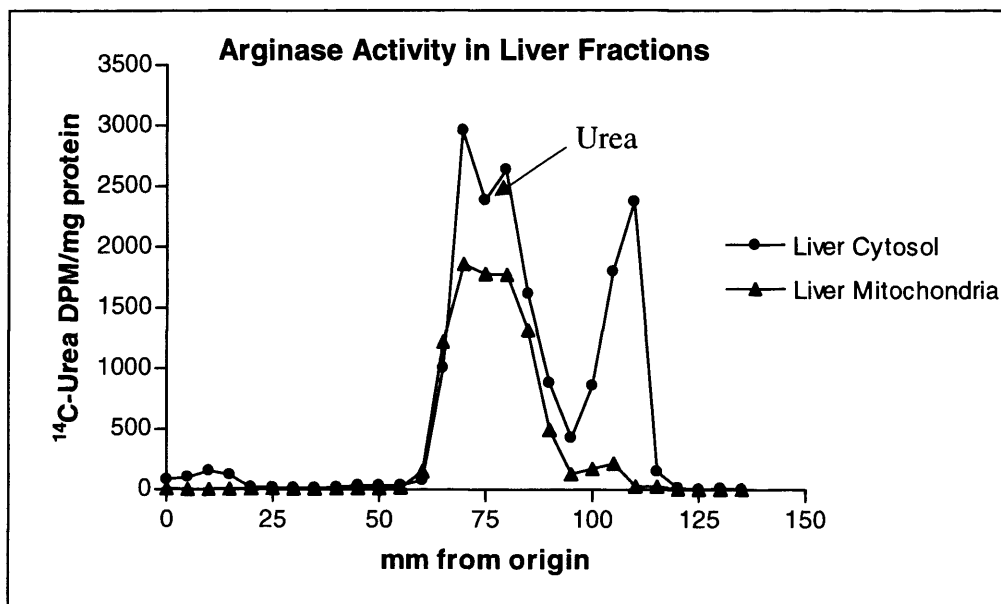


Figure 4.4: Arginase activity in subcellular fractions of the liver. There was arginase activity in both fractions, ( $n=2$ , repeated twice).

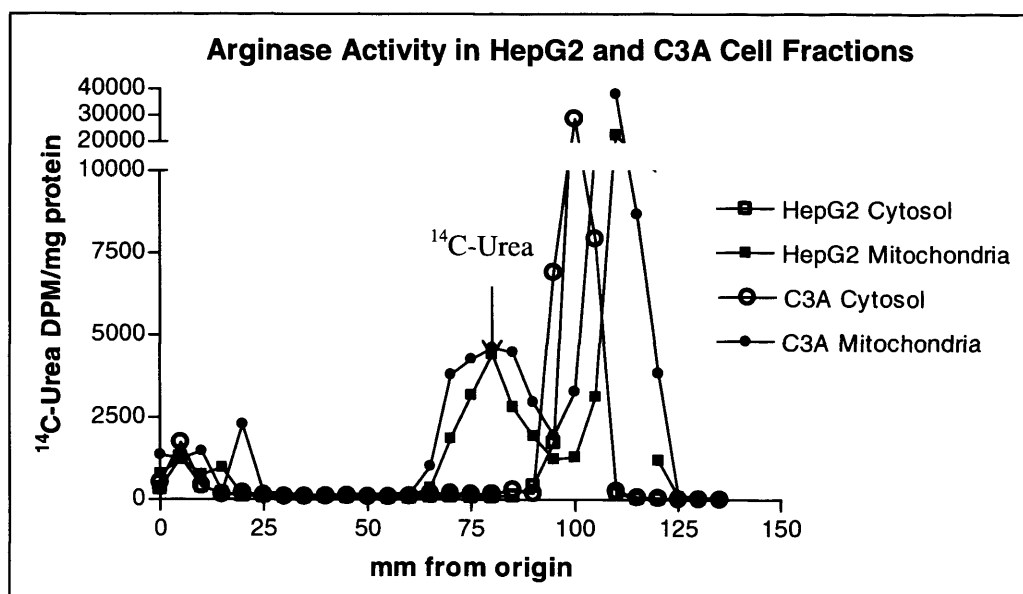


Figure 4.5: Arginase activity in subcellular fractions of HepG2 and C3A cells. There was arginase activity in the mitochondrial fractions of both HepG2 and C3A cells. The cytosolic fraction however, contains no arginase activity, ( $n=2$ , repeated twice).

#### 4.1.2.2 Arginase I Protein Expression

Western blots for arginase I were carried out. These showed that the protein was undetectable in both HepG2 and C3A cells, whereas it is evident in primary human hepatocytes, as expected, and not in the rat kidney biological negative control (Figure 4.6, page 91). Two isoforms of arginase I are detected (113).

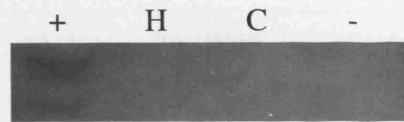


Figure 4.6: Arginase I protein expression in hepatocytes (+) but not in HepG2 (H) and C3A cells (C), or the negative control rat kidney (-), (repeated 3 times).

#### 4.1.2.3 Arginase II Protein Expression

Western blots for arginase II also showed increased expression in HepG2 and C3A cells in comparison to human liver (Figure 4.7). Rat kidney was used as an arginase II positive control where it is highly expressed as the antibody has cross-species reactivity (114).

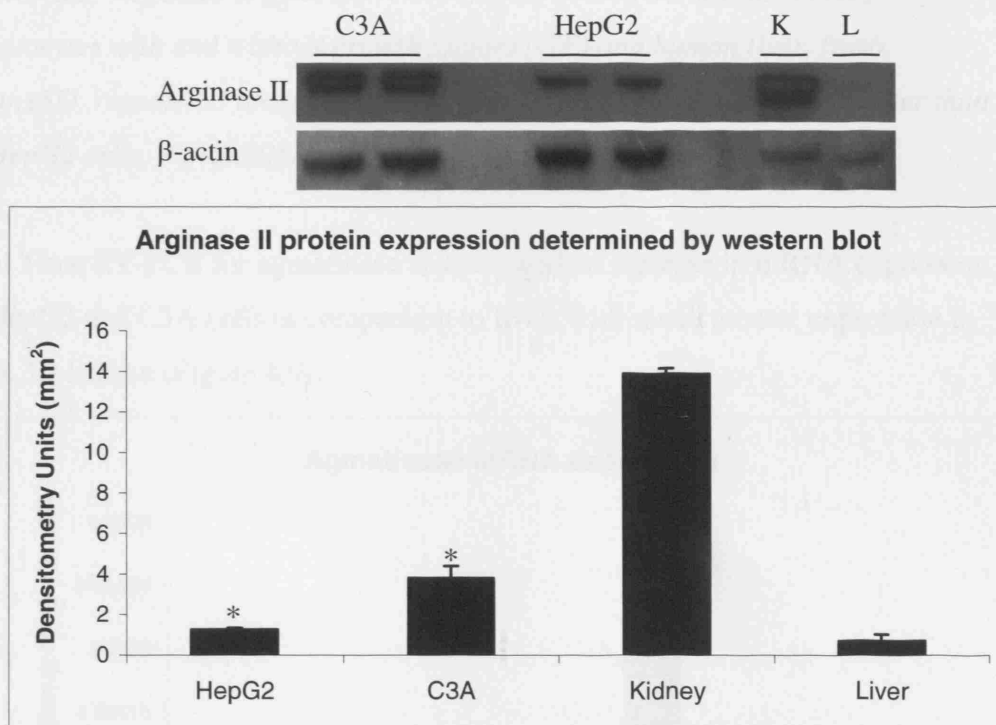


Figure 4.7: Western blots and densitometry for arginase II. Protein is expressed in C3A at significantly greater levels than in HepG2 cells (\* $p < 0.05$ ), ( $n=2$ , repeated twice) and HepG2 cells. Arginase II is also evident in the rat kidney positive control (K) and the liver control (L). Lanes were equally loaded with 40  $\mu$ g protein.  $\beta$ -actin was used to normalise densitometric data.

#### 4.1.2.4 Arginase II, Agmatinase and ADC mRNA Expression

Real Time RT-PCR for arginase II showed a marked increase in mRNA expression in HepG2 and C3A cells in comparison to liver, with high levels in C3A 3D culture (Figure 4.8, page 92).

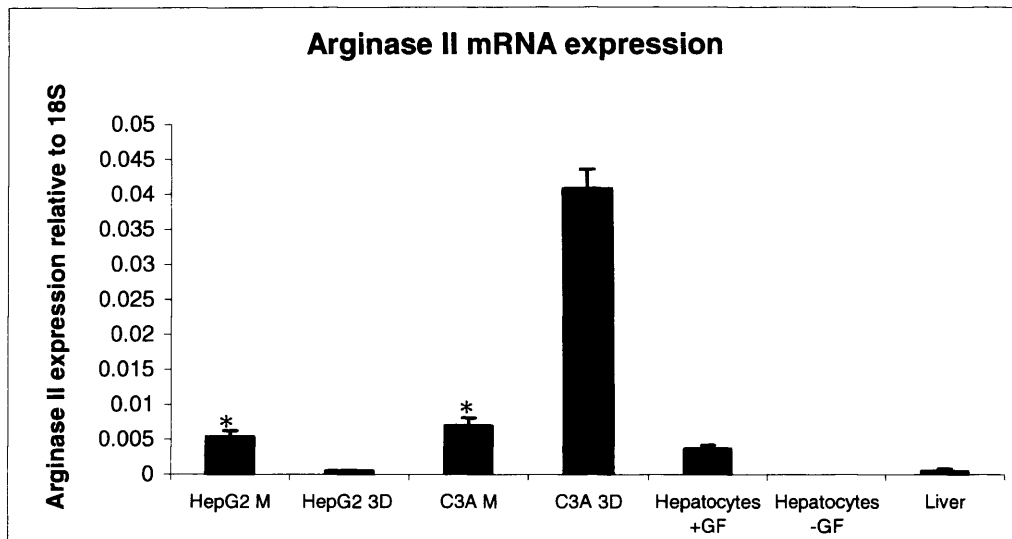


Figure 4.8: Arginase II gene expression levels in HepG2 and C3A cells, hepatocytes with and without growth factors (GF) and human liver, (n=6, mean±SD, repeated 3 times). Expression in C3A cells is significantly higher than in HepG2 cells, \* $p<0.005$ .

Real Time RT-PCR for agmatinase also showed an increase in mRNA expression in HepG2 and C3A cells in comparison to liver, with much greater expression in C3A 3D culture (Figure 4.9).

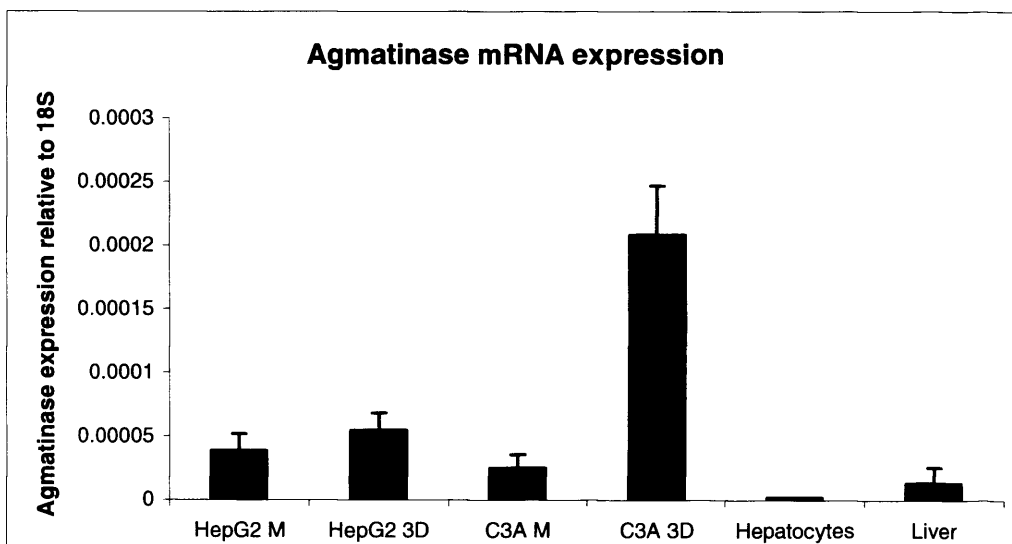


Figure 4.9: Agmatinase gene expression levels in HepG2 cells and C3A monolayer cells where it is slightly higher than in hepatocytes and whole human liver (n=6, mean±SD, repeated 3 times).

Unfortunately, after a number of attempts, the Real Time RT-PCR for ADC was unsuccessful.

#### 4.1.2.5 Arginase Inhibition assay

To determine the degree of contribution to urea production by arginase II, arginase inhibitor studies using nor-NOHA, could inhibit the conversion of  $^{15}\text{N}_2$ -guanido-arginine to  $^{15}\text{N}$ -urea in cell lysates. Figure 4.10 shows that the addition of nor-NOHA to cell lysates inhibits arginase II activity in a concentration dependent manner, with complete ablation evident at 200 $\mu\text{M}$ .

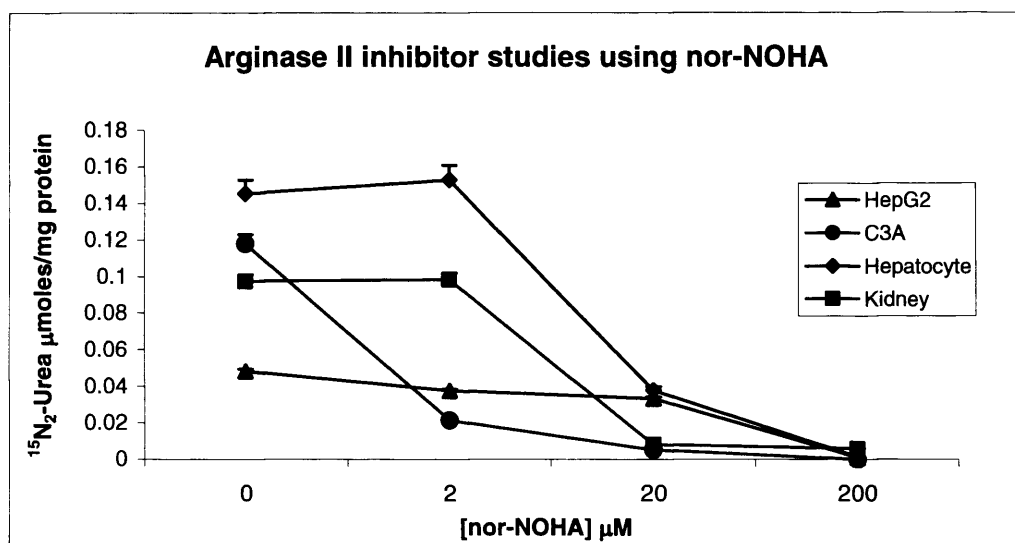


Figure 4.10: Arginase II inhibitor studies using nor-NOH. As the concentration of the arginase inhibitor increases (0-200 $\mu\text{M}$ ), production of  $^{15}\text{N}$ -labelled urea in all cell types decreases ( $n=3$ , mean $\pm$ SD).

#### 4.1.3 Discussion

The results from this chapter show that neither HepG2 nor C3A cells express arginase I protein, as shown by the cell fractionation studies and the Western blot. This correlates with the findings of chapter 3, that real time RT-PCR studies showed no arginase I mRNA expression. The arginase activity seen in chapter 3 can now be attributed to arginase II activity. This is confirmed not only by the presence of mRNA and protein expression, but also arginase activity in the mitochondrial fractions of HepG2 and C3A cells. In addition, the inhibitor studies show that the majority of urea produced from arginase was due to arginase II activity. Whilst higher expression of agmatinase was observed in these cells, the direct link to urea production via ADC could not be established.

Together with data from chapter 3, which showed that both HepG2 and C3A cells are



incapable of *de novo* urea synthesis from  $^{15}\text{NH}_4\text{Cl}$ , these results shows that urea detected in medium from C3A cells in particular is not due to ammonia detoxification via the urea cycle. Urea is synthesized independently of the urea cycle in C3A cells and to a far lesser extent in HepG2 cells. Expression levels of arginase II mRNA and protein expression were significantly different in the two cell lines, both were expressed at lower levels in HepG2 cells than in C3A cells (Table 4.4). This may account for the difference as regards perceived urea cycle function in HepG2 and C3A cells.

*Table 4.4: Comparison of arginase II expression in C3A and HepG2 cells. Data are presented as mean $\pm$ SD, with significance values included.*

	C3A	HepG2	p value
Urea ( $\mu\text{moles/mg protein}$ )	0.045 $\pm$ 0.005	0.004 $\pm$ 0.007	***<0.0001
$^{15}\text{N}$ -Urea ( $\mu\text{moles/mg protein}$ )	0.005 $\pm$ 0.0008	0.003 $\pm$ 0.005	ns
Arginase II mRNA expression (Copies relative to 18S)	6.98 $\pm$ 1.12( $\times 10^{-5}$ )	5.36 $\pm$ 0.88( $\times 10^{-5}$ )	**<0.005
Arginase II protein expression (densitometry units $\text{mm}^2$ )	3.82 $\pm$ 0.6	1.27 $\pm$ 0.08	*<0.05

#### 4.1.4 Conclusion

Whilst HepG2 cells are known to exhibit minimal urea cycle function, C3A cells are reported to produce urea, and this has been assumed to be due to a functional urea cycle since it is a hepatic cell line (38;87-89;115). It was clearly demonstrated in Chapter 3 that HepG2 and C3A cells do not possess a functional urea cycle. In addition to this, the data presented in chapter 4, show conclusively that ammonia cannot be specifically removed by this pathway and converted to urea. Moreover, in C3A cells, urea is produced solely from the action of arginase II on arginine, a urea-cycle independent mechanism not involved in ammonia detoxification. Therefore these cells do not provide the ammonia detoxificatory capacity required for liver support. This has significant implications for BAL devices which utilize C3A cells, one device, which has undergone clinical trial, showed no significant improvement in ammonia levels (38), despite this a multi-centre trial is currently in progress.

An alternative method of ammonia detoxification has been considered by other groups. This has been to increase GS activity in HepG2 cells by gene transfer (116;117), as this

may be a preferred pathway of ammonia detoxification for a hepatoma cell line (48;118). However, as it has not yet been firmly established whether increased glutamine, in addition to raised levels of circulating ammonia, contribute to hepatic encephalopathy, which is a serious manifestation of liver failure, the pathway of ammonia removal in a BAL is important.

Restoring the urea cycle in the HepG2 cell line and ensuring adequate ammonia detoxification via this pathway is therefore essential. These investigations are described in Chapter 5.

## Chapter 5: Restoring Urea Cycle Function in HepG2 Cells

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As shown in Chapter 3, neither HepG2 nor C3A cells have a functional urea cycle due to the down-regulation of two urea cycle genes, OTC and arginase I, although, they do express some CPSI, AS and AL. It has been shown by others that HepG2 cells may preferentially detoxify ammonia by converting it to glutamine via GS. This would be characteristic of a perivenous, as opposed to periportal, hepatocyte phenotype. Others have developed a cell line by gene transfer of GS, which resulted in increased ammonia metabolism into glutamine (116). Whilst the role of raised ammonia levels to hepatic encephalopathy, a serious manifestation of liver failure, has been established, the contribution of glutamine is still under dispute. It is postulated that it would be more effective to reinstate the urea cycle, and maintain the 'normal' pathway of ammonia detoxification, and these investigations consisted of the following three approaches:

1. **Demethylating the urea cycle genes:** It is well established that methylation silences many key genes in cancer cells, therefore HepG2 cells were incubated with the demethylating agent 5-azacytidine. Real Time RT-PCR was then carried out to determine whether OTC and arginase I were re-expressed. In addition, a real time RT-PCR was carried out for HNF-4 expression as this transcription factor is involved in regulating expression of hepatocyte specific genes.
2. **Adaptation to arginine deficient medium:** HepG2 cells were adapted to medium deficient in arginine in an attempt to restore urea cycle function, a hypothesis based on previous findings with OTC deficient rat hepatoma cell lines (119).
3. **Multiple gene transfer:** Plasmid vectors containing OTC and Arginase I were constructed and transfected into HepG2 cells to determine whether this method could lead to ammonia detoxification and urea production via the urea cycle.

## 5.1 Demethylation of the Urea Cycle Genes

Methylation is a process by which gene expression is regulated. Discovered over 40 years ago, the presence 5-methylcytosine in DNA has since been associated with the regulation of gene expression and is an important mediator in the repression of gene expression during mammalian development (120;121). In normal cells, methylation occurs mainly at CpG poor regions, whereas CpG rich regions seem to be protected (122). Initial experiments that depicted this phenomenon used methylation sensitive restriction enzymes to show that expression of tissue specific genes correlated with hypomethylation (123). One example of this is seen with the liver specific OTC gene, where minimal expression in the kidney is due to hypermethylation. Housekeeping genes employ the same mechanism of expression, in that the 5' region is CpG rich and completely unmethylated in all tissues when active (124). Further transfection experiments and *Xenopus* oocyte microinjections demonstrated that methylation inhibits gene expression (125). Therefore DNA methylation modifies the eukaryotic genome in order to repress transcription. Some other processes in which this method of genetic silencing occurs include X-chromosome inactivation, carcinogenesis and genomic imprinting (126-131).

It has been well documented that many cancers have aberrant methylation in the promoter region of various genes that result in silencing: examples include, tumour suppressor genes, genes involved in DNA repair, cell growth and differentiation, amongst others (36;132;133). However, in tumourigenesis the converse is also true, in that reduced methylation of genes such as *H-ras*, responsible for controlling cell division, has also been observed (134). Mechanistically, repression of transcription can be brought about as DNA methylation can sterically hinder the binding of transcription factors to gene promoters. An alternative process by which transcription is directly repressed involves the active binding of methyl CpG binding domain proteins to methylated DNA (122). These can also recruit transcription co-repressors such as histone deacetylases. A third mechanism comprises DNA methyl transferase proteins that non-enzymatically repress transcription, and, like methyl CpG binding domain proteins, also recruit histone deacetylases (121;135). These methods result in gene silencing by either directly repressing transcription or by aiding the formation of repressive chromatin structures on to DNA (136;137).

Further evidence that methylation is involved in the suppression of gene expression has been provided via the use of the demethylating agent 5-aza-2'-deoxycytidine. Many studies have shown that genes silenced in cultured cell lines can be reactivated upon exposure to this demethylating agent. 5-azacytidine and the commercial 5-aza-2'-deoxycytidine (Decitabine) are cytosine analogues that are incorporated into DNA (138;139). They cause multiple changes in cell physiology and are very toxic to cells (138;140). Once these analogues are incorporated into the DNA there is a rapid loss of the methylation status of the DNA, and a perturbation of the mechanisms that maintain the methylation status. Since DNA methyl transferase enzymes become covalently and irreversibly bound to the cytosine analogues, there is a decrease in total DNA methyltransferase activity (140), and along with decondensation of the chromatin structure and alteration in replication timing, this leads to the eventual demethylation of genomic DNA (141).

Previous studies on OTC expression in rat hepatoma cell lines have shown that hypermethylation is responsible for silencing the OTC gene (142;143). In order to investigate whether methylation does indeed have a role to play in the silencing of the OTC and arginase I genes in HepG2 cells, experiments were conducted in which HepG2 cells were exposed to varying concentrations of 5-azacytidine. The effects on gene expression were observed by using Real Time RT-PCR analysis.

### **5.1.1 Methods**

#### **5.1.1.1 Culturing HepG2 cells with the demethylating agent 5-azacytidine**

##### *Materials*

5-azacytidine, 1mM stock in DMSO (Sigma)

12 well plates, for monolayer culture of HepG2 cells

Microcentrifuge

##### *Methods*

HepG2 cells were cultured in 5-azacytidine for 72 hours. Culture conditions were as previously described in Chapter 2.0 with the following modifications. For monolayer culture, HepG2 cells were plated in 12-well plates at a density of 60,000 cells/ml/well and incubated at 37°C for 48 hours after which 0, 5, 10, 20 and 40 µM 5-azacytidine final concentration was added to 1ml fresh medium. After 48 hours, the medium was changed and replaced with 1ml fresh medium with the same concentration of 5-azacytidine added.

After a further 24 hours the cells were harvested for RNA extraction and cDNA synthesized. GC-MS for urea was carried out as previously described (Chapter 3, section 3.1.1.2, page 61). Real Time RT-PCR was carried out for OTC and arginase I (section 3.2, page 71).

In addition, mRNA expression for HNF-4 was determined using the following primers:

HNF-4 forward 5'- CGGAGCTGGCGGAGAAGAG

HNF-4 reverse 5'- AGATGATGGCTTTGAGGTAGG

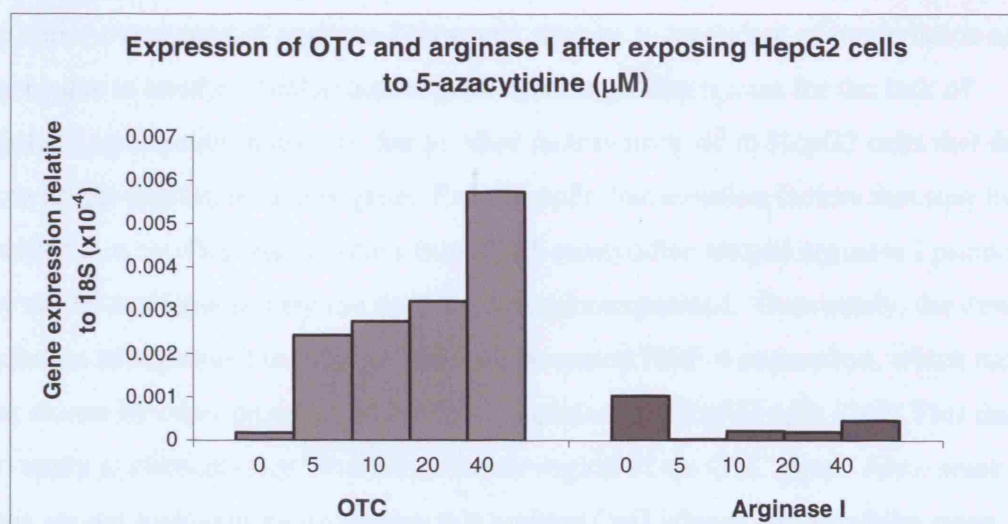
and the cycling conditions: 95°C 15', {95°C 20'', 60°C 20'', 72°C 30''} x 40 cycles, and 60°C 30''.

## 5.1.2 Results

Urea could not be detected by GC-MS in conditioned medium samples from 5-azacytidine treated HepG2 cells, above background.

### 5.1.2.1 OTC and Arginase I mRNA Expression

Real Time RT-PCR results indicate that after exposing cells to the demethylating agent, OTC mRNA expression increases, however the same is not seen for Arginase I mRNA expression (Figure 5.1).



*Figure 5.1: OTC and Arginase I expression in HepG2 cells exposed to 5-azacytidine. OTC expression increases with 5mM 5-azacytidine, and then remains high, arginase I however is unaffected by the presence of the demethylating agent, (n=2, repeated twice).*

### 5.1.2.2 HNF-4 expression

Expression of HNF-4 in HepG2 cells is greater than in the human liver (Figure 5.2).

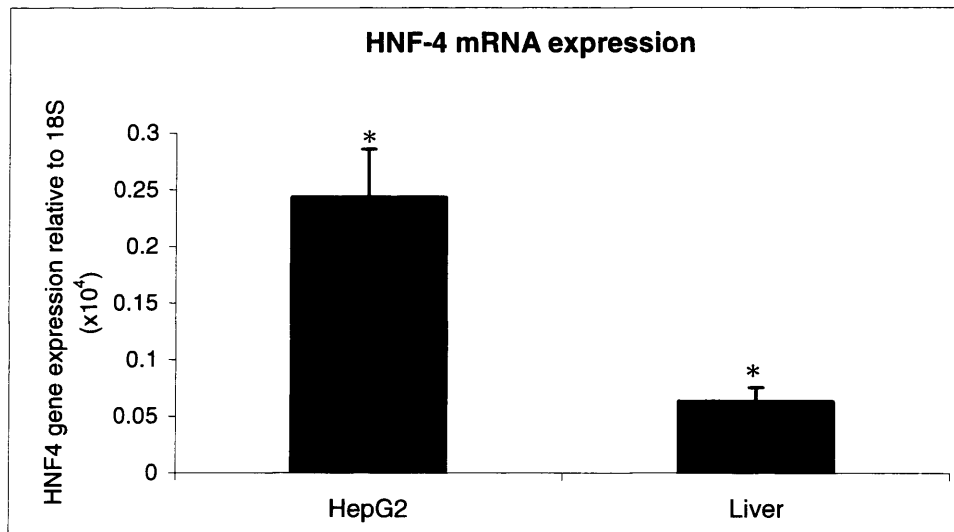


Figure 5.2: *HNF-4 mRNA expression levels in HepG2 monolayer cells in comparison to human liver, (n=6, mean  $\pm$ SD, repeated twice, \* $p$ <0.005).*

### 5.1.3 Discussion

The results show that in response to treatment with the demethylating agent 5-azacytidine, mRNA expression of OTC increases, which is not the case with arginase I. This suggests that down-regulation of OTC may be due to promoter hypermethylation. The under-expression of arginase I however, may be independent of methylation and may be due to another mechanism of gene silencing. One reason for the lack of arginase I up-regulation may be due to other factors intrinsic to HepG2 cells that do not allow for up-regulation of this gene. For example, transcription factors that may be involved in activating transcription from the 5-azacytidine treated arginase I promoter may not be available as they too may not be under-expressed. Conversely, the down-regulation of arginase I may be an effect of increased HNF-4 expression, which has been shown by other groups, and is highly expressed in HepG2 cells (144). This may also apply to elements that bind the enhancer region of the OTC gene. Also, some genes are not regulated by promoters that contain CpG islands and so whilst some experiments have shown that 5-azacytidine can regulate gene expression indirectly of the promoter regions, this is not always the case. Promoter hypermethylation seems to be responsible for the under-expression of OTC but not of Arginase I, as mRNA expression of this gene was not increased upon treating cells with the hypomethylating agent 5-azacytidine.

## **5.2 Adaptation to Arginine Deficient Media**

A number of studies have shown that urea cycle activity can be up-regulated by adapting urea cycle deficient cells to arginine deficient media (119;142;145). Another characteristic of HepG2 cells, as well as other hepatoma cell lines, is increased glutamine synthesis (48;118). The radio-labelled studies carried out previously in this chapter may suggest this in the HepG2 cells currently used for the bioartificial liver being developed in the group. The problem with an increase in glutamine synthesis is the fact that in high levels it is toxic to the brain (45). So the purpose of this study is to adapt cells to arginine deficient medium thereby forcing them to make arginine via the urea cycle and thus reducing the amount of ammonia available to convert glutamate to glutamine.

### **5.2.1 Methods**

#### **5.2.1.1 Culturing HepG2 cells in arginine deficient medium**

##### *Materials*

Components, see Table 5.1 (page 102)

Basal Medium

Fischer's Medium

50ml Nunc tubes

50ml Syringe

200µm filters

Sterile water

1mg/ml arginine

1mg/ml L-ornithine

1mg/ml citrulline

1mg/ml putrescine

##### *Method*

The arginine-free medium was made by adding all the components in Table 5.1, to sterile water, adjusted to pH7.4, filter sterilised and stored at 4°C. Before use, each 50ml aliquot was supplemented with the supplements added to α-MEM media, see Chapter 2 General Methodology Table 2.4 page. HepG2 cells were grown as usual in monolayer culture (standard α-MEM, arginine 126mg/L) but adapted over time to the two types of arginine low medium: i) Basal medium (arginine 21mg/L) and ii) Fischer's medium (arginine 15mg/L), which were supplemented as for α-MEM. The adapting process was carried out by using the following



combinations of medium with each step maintained for 3 passages or until cells looked healthy:

- 25% normal  $\alpha$ MEM: 75% arginine low medium,
- 50% normal  $\alpha$ MEM: 50% arginine low medium,
- 75% normal  $\alpha$ MEM: 25% arginine low medium

This process was also conducted with arginine-deficient medium supplemented with 5mg/L arginine, 10mg/L L-ornithine, 50mg/L citrulline and 0.3mg/L putrescine.

*Table 5.1: Components to make arginine free media.*

<i>Component</i>	<i>Final mg/L</i>	<i>Component</i>	<i>Final mg/L</i>
CaCl <sub>2</sub> .2H <sub>2</sub> O	264	L-Lysine.HCl	146
KCl	400	L-Methionine	30
MgSO <sub>4</sub> .7H <sub>2</sub> O	200	L-Phenylalanine	66
NaCl	3500	L-Serine	42
NaHCO <sub>3</sub>	3700	L-Threonine	95
NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	141	L-Tryptophan	16
D-Glucose	4500	L-Tyrosine	72
Phenol Red	15	L-Valine	94
Sodium Pyruvate	110	D-Ca Pantothenate	4
DL-68Thioctic Acid	0.2	Choline Chloride	4
L-Alanine	25	Folic Acid	4
L-Cystine	48	i-Inositol	7.2
Glycine	30	Niacinamide	4
L-Histidine HCl.H <sub>2</sub> O	42	Pyridoxine HCl	4
L-Isoleucine	105	Riboflavin	0.4
L-Leucine	105	Thiamine HCl	4

#### 5.2.1.2 OTC Specific Activity Assay

##### *Materials:*

Mitochondrial lysis buffer (0.5% Triton X-100, 10mM HEPES in water)

Ornithine solution (7.5mM Ornithine, 405 mM Triethanolamine, in water, pH8)

150mM Carbamoyl Phosphate Lithium Salt

Ornithine Transcarbamylase (OTC) stock solution 0.1U/ $\mu$ l

Acid Stop solution: 3:1 Phosphoric Acid : Sulphuric Acid

Colour developer: 3% 2,3-butanedionmonoxime in water

Sanyo MSE Soniprep 150

37°C water bath

105°C heating block

Spectrophotometer Uvikon 930 set at 490nm

1.6ml disposable cuvettes

### Method:

To assess OTC specific activity, HepG2 cells were washed twice with cold 1x PBS. Cell lysis buffer was then added to the cells and the plate incubated on ice for 20 minutes, the cells were then scraped off and sonicated at 10 microns for 10 seconds on ice. A standard curve was prepared by diluting the OTC stock solution 1:100 and then using 0, 1, 2, 5, 10 $\mu$ l made up to 50 $\mu$ l in cell lysis buffer to give 0.001, 0.002, 0.005 and 0.01 OTC U/ $\mu$ l respectively. The 1:100 dilution was made fresh each time.

For OTC specific activity of cell homogenates, to 1.5ml eppendorfs, 50 $\mu$ l homogenate, 500 $\mu$ l ornithine solution and 125 $\mu$ l water were added. At 10 second intervals 75 $\mu$ l carbamoyl phosphate was added to each tube and the reaction incubated at 37°C for 10 minutes. To stop the reaction 375 $\mu$ l acid stop solution was added, then 47 $\mu$ l colour developer, and the tubes were gently vortexed briefly. The lids were pricked and the tubes were placed in a 100°C heating block in a fume hood covered with foil for 15 minutes. The samples were cooled to room temperature for 10 minutes, again covered in foil, and then read on spectrophotometer at 490nm. The background control was prepared by adding the substrates to the stop solution, followed by the homogenate and colour developer and the sample boiled immediately. Protein was quantified using the Bradford assay (Chapter 2, section 2.1.5, page 50).

### 5.2.2 Results

Data show that upon reducing the levels of arginine in the media OTC specific activity increases (Figure 5.3). Cells could not be adapted to arginine-deficient medium.

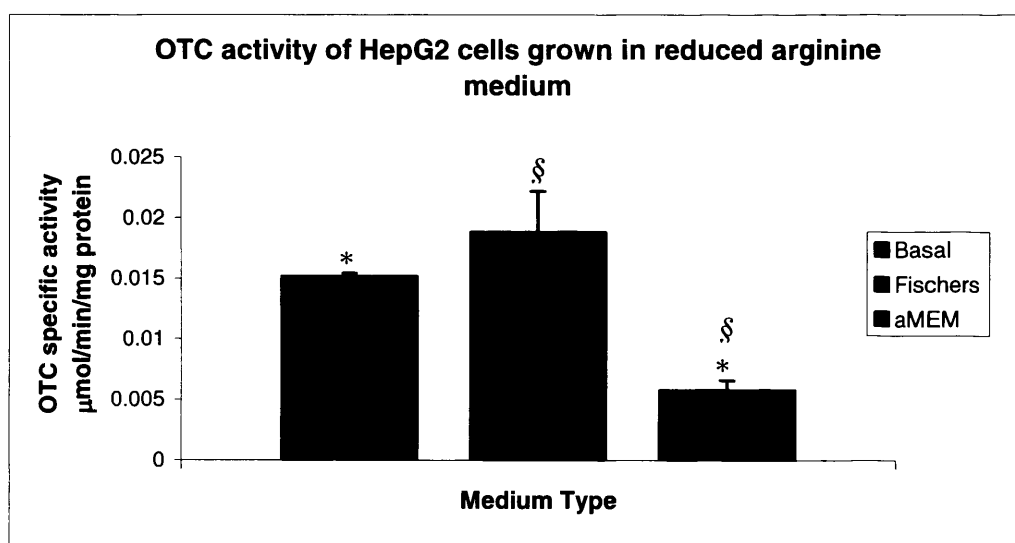


Figure 5.3: HepG2 cells grown in Basal, Fischer's, and normal  $\alpha$ MEM medium, ( $n=3$ , mean $\pm$ SD, \* $p<0.005$ , § $p<0.005$ , there was no significant difference between Basal and Fischer's medium).

### **5.2.3 Discussion**

Despite an apparent increase in OTC activity in the cells grown in arginine low medium, this medium was detrimental to cell growth, even though the adaptation to both arginine low and arginine free medium was done over a long period of time. Eventually, phenotypically, the cells looked very poor, with many vacuoles, and they had stopped growing. One possible reason for this could be that since arginine is essential to growth, the HepG2 cells may not have been able to make enough arginine since they were unable to up-regulate OTC sufficiently for the purposes of cell maintenance and proliferation.

## **5.3 Multiple Gene Transfer to Restore Urea Cycle Function in HepG2 Cells**

Since CPSI, AS and AL are expressed to some degree in HepG2 cells, it was hypothesized that that by replacing OTC and arginase I, some urea cycle function could be restored. This was carried out by the following:

1. Creating pcDNAZeoOTC and pcDNAHygArgI:
  - i. Designing cloning primers for the OTC and arginase I sequences.
  - ii. Amplifying OTC and arginase I cDNA from human liver.
  - iii. Inserting OTC and arginase I into pcDNAZeo and pcDNAHyg.
  - iv. Sequencing pcDNAZeoOTC and pcDNAHygArgI.
2. Creating Urea Cycle Expressing HepG2 Cells:
  - i. Transiently transfecting HepG2 cells with pcDNAZeoOTC and pcDNAHygArgI.
  - ii. Developing stable transfectants.
  - iii. Assessing urea cycle function in the transfectants.

### **5.3.1 Methods: Creating pcDNAZeoOTC and pcDNAHygArgI**

A multiple gene transfer approach was taken to restoring urea cycle function in HepG2 cells. OTC and arginase I cDNA, amplified from normal human liver, were inserted into the pcDNA3.1 vectors, pcDNAZeo and pcDNAHyg respectively. Two different antibiotic resistance plasmids were used in order to be able to select for HepG2 cells

transfected with both OTC and arginase I.

### 5.3.1.1 Designing cloning primers for OTC and Arginase I

The cloning primers were designed to contain restriction sites that would enable the construct to be inserted into the multiple cloning site of the pcDNA3.1 vectors. Vector NTI version 10 software was used to design the primers. The Gen bank sequences were uploaded for OTC (NM\_000531) and Arginase I (NM\_000045). A Kozak initiation sequence was added to the forward primers. Then a restriction site was added to the beginning of the forward primers and the end of the reverse primers. Semi-directional cloning was used, with both forward primers containing a blunt-end EcoRV restriction site, and both reverse primers containing an overhang ApaI restriction site (Figure 5.4).

#### OTC cloning Forward

5'- ctgatatcccac**at**gctgtttaatctgagg -3'

#### OTC cloning Reverse

5'- gtgggccccacatcaaaatttaggcttctg -3'

#### ArginaseI cloning Forward

5'- tggatatcccac**at**gagcgccaagtccaga -3'

#### ArginaseI cloning Reverse

5'- gtgggcccttacttaggtgggtaaggta -3'

*Figure 5.4: The OTC and Arginase I cloning primers. Restriction sites (underlined) and a Kozak sequence (italics) were incorporated into the primers. Start and stop codons are highlighted (bold).*

### 5.3.1.2 Amplifying OTC and Arginase I cDNA from human liver

In order to successfully amplify OTC and arginase I cDNA without introducing errors into the sequence, a high fidelity proofreading polymerase was used.

#### Materials

RNAagents Total RNA Isolation System

RevertAid First strand cDNA Synthesis Kit

Accuprime Pfx Supermix (Invitrogen)

OTC and Arginase I cloning primers

Sterile water

Scalpel

1.5 ml microfuge tubes

QIAquick Gel Extraction kit (Qiagen)

UV light box

## NanoDrop

### *Method*

RNA was isolated from human liver and cDNA synthesized as described under 2.0 General Methods page. To amplify the cDNA, the high fidelity Accuprime polymerase mix was used, and the reaction assembled by adding to 0.2ml PCR tube 22.5µl polymerase, 200nM of reverse and forward primers and 1µl cDNA. The PCR conditions were as follows, 95°C 5 minutes, (95°C 15 seconds, 55°C 30 seconds 68°C 1.5 minutes) x30 cycles, hold 4°C. This was done in triplicate for both OTC and arginase I. The PCR reactions were run on a 1% agarose gel using Hyperladder I as marker. The OTC and arginase I bands were visualized on a UV light box and quickly excised into autoclaved microfuge tubes. DNA was extracted from the gel according to the QIAquick Gel Extraction kit protocol. Briefly the gel was solubilized and the liquid added to a DNA binding column. After washing, the DNA was eluted into 25µl Tris-Cl buffer (supplied with kit).

### **5.3.1.3 Restricting pcDNA3.1 and HepG2OTC cDNA with ApaI and EcoRV**

#### *Materials*

(Enzymes from Promega, supplied with restriction buffer and BSA)

Restriction enzyme ApaI 10U/µl, (no more than 10% of final volume)

Restriction enzyme EcoRV 10U/µl, (no more than 10% of final volume)

10x Restriction Buffer, 5µl (1x final concentration) (supplied with the enzyme)

10mg/ml Acetylated BSA, 5µl (1mg/ml final concentration)

1µg pcDNA3.1Hyg (Invitrogen)

1µg pcDNA3.1Zeo (Invitrogen)

OTC cDNA from Accuprime PCR

Arginase I cDNA from Accuprime PCR

Sterile distilled water

0.2ml PCR tubes

QIAquick Gel Extraction kit

Water bath

#### *Method*

For the 5' blunt ended restriction site, EcoRV was first used. 0.2ml PCR tubes were set up containing the above reagents and either pcDNAHyg, pcDNAZeo, OTC or arginase I. The DNA was incubated with the enzyme at 37°C for 2 hours and then cleaned using a QIAquick Gel Extraction kit according to the manufacturers' protocol. The DNA was

eluted from the columns with 25µl Tris-Cl buffer. For the 3' overhang restriction site using *ApaI*, this process was repeated with incubation at 30°C for 2 hours. After the restriction the DNA was cleaned in the same way and eluted.

#### **5.3.1.4 De-phosphorylating Overhang and Blunt Ends of Restricted pcDNA3.1**

The ends of the restricted pcDNA vectors were dephosphorylated to prevent self-ligation during subsequent steps.

##### *Materials*

Alkaline Phosphatase (0.1U/µl) (Promega)

0.5M EDTA pH7.5

0.2ml PCR tubes

QIAquick Gel Extraction kit (Qiagen)

37°C Water bath

56°C heating block

##### *Method*

To the cleaned 25µl of pcDNAHyg and pcDNAZeo, 5µl 10x reaction buffer and 5µl alkaline phosphatase (0.01U/µl) were added and made up to 50µl with water. These were incubated at 37°C 15 minutes then 56°C 15 minutes. A second aliquot of enzyme was added and the incubations repeated. The reaction was stopped by adding 1µl EDTA. The DNA was cleaned using the QIAquick Gel Extraction kit.

#### **5.3.1.5 Ligating Vectors and cDNA**

##### *Materials*

T4 DNA Ligase kit (Promega)

0.5ml microfuge tubes

##### *Method*

The pcDNAZeo and OTC cDNA, and pcDNAHyg and arginase I cDNA were ligated together using T4 DNA ligase by mixing 100ng vector DNA and 72ng insert cDNA with ligase buffer (1x final concentration from 10x stock) and 1unit T4 DNA ligase; this was incubated at room temperature for 3 hours, after which time the products were used to transform competent *E.coli*.

#### **5.3.1.6 Transforming *E.coli* with pcDNAZeoOTC and pcDNAHygArgI Constructs**

##### *Materials*

XL10-Gold Ultracompetent cells (Stratgene)

LB Agar containing 100µg/ml ampicillin (for 1L: 5g NaCl, 2.5g yeast extract, 5g tryptone, 10g agar, pH7.0 with 5M NaOH autoclave 121°C, 20 minutes)  
SOC broth (for 1L: 0.5g NaCl, 5g yeast extract, 20g tryptone, SDW to 950ml, add 10ml 250mM KCl, autoclave, add 20ml 1M glucose filter sterilized and 5ml autoclaved 2M MgCl<sub>2</sub>)  
1.5ml microfuge tubes  
42°C water bath  
37°C shaker  
37°C incubator  
PCR reagents (as described in Chapter 2, section 2.2.3, page 53)

#### *Method*

LB agar was made, 100µg/ml ampicillin added, plates poured and dried upside down in a 37°C incubator. For the transformation, the manufacturers' protocol was followed. The 100µl *E.coli* aliquots were thawed on ice, 4µl β-mercaptoethanol added, and incubated on ice for 10 minutes, swirling every 2 minutes. The *E.coli* were then aliquotted into 20µl per prechilled microfuge tube, 2µl of ligation mix was added and the tubes were incubated on ice for 30 minutes. The positive control was (pUC) plasmid (supplied) and the negative control was *E.coli* without ligation mix. SOC broth was preheated to 42°C. The tubes were heat-pulsed in the water bath at exactly 42°C for exactly 30 seconds. 200µl of SOC broth was added to each microfuge tube, and these were incubated at 37°C for 1 hour with shaking at 225rpm. After this time, 200µl was spread onto the agar plates and these incubated at 37°C overnight.

#### **5.3.1.7 Identifying Positive Colonies Containing pcDNAZeoOTC and pcDNAHygArgI**

The day after plating, the bacterial colonies were checked to see if they contained the pcDNAZeoOTC and pcDNAHygArgI constructs, as seen by discrete white colonies on the agar.

#### *Materials*

PCR mix  
Primers 100µM stock  
LB Agar plates with 100µg/ml ampicillin

#### *Method*

A PCR was set up, as in chapter 2.0 using primers that span the multiple cloning site (MCS) (forward 5'agcagagctctctggctaactagagaaccc, reverse 5'tggcaccttcagggtcaaggaag).

The PCR mix was aliquotted 20µl per 0.2µl PCR tube. A selection of colonies, usually 10-20 depending on how many had grown, were picked individually, streaked onto the new LB agar plates using a 20µl pipette tip, and then the tip was inserted into the PCR mix. The PCR was performed as below and products run on a 1% agarose gel.

95°C 15 minutes,	
94°C 30 seconds	} x30 cycles
55°C 30 seconds	
72°C 1.5 minutes	
72°C 10 minutes	

#### **5.3.1.8 Miniprep of E.coli Containing the pcDNAZeoOTC and pcDNAHygArgI Constructs and Sequencing**

##### *Materials*

LB broth containing 100µg/ml ampicillin (for 1L LB 5g NaCl, 2.5g yeast extract, 5g tryptone, pH7.0 with 5M NaOH autoclave 20 minutes 15lb/sq)

37°C shaker

50ml tubes

Miniprep plasmid extraction kit (Qiagen)

Glycerol

1.5ml and 2ml microfuge tubes

Micro centrifuge

Qiagen Sequencing Services

NanoDrop

##### *Method*

Positive colonies, as shown by the PCR were picked off the streaked plates, 5ml LB broth inoculated incubated at 37°C in a shaker overnight, but for no longer than 17 hours. The following day, 2ml of each culture, in duplicate, was centrifuged at 16000xg for 1 minute. From the remaining culture 0.85ml was mixed well with 0.15ml glycerol and stored at -80°C. The construct was isolated from the pellet according to the miniprep plasmid extraction kit protocol. The amount of DNA was quantified using the NanoDrop and 0.5mg of each miniprep was sent for sequencing.



### **5.3.2 Methods: Creating Urea Cycle Expressing HepG2 Cells**

#### **5.3.2.1 Transient Transfection of HepG2 cells with pcDNAZeoOTC and pcDNAHygArgI**

HepG2 cells were transfected with the constructs pcDNAZeoOTC and pcDNAHygArgI using Lipofectamine 2000 reagent. Transfection efficiency was assessed using a pcDNA3.1LacZ control.

##### *Materials*

Lipofectamine 2000 (Invitrogen)

$\alpha$ -MEM medium, serum free and antibiotic free

$\alpha$ -MEM complete medium, without antibiotics

##### *Method*

HepG2 cells were plated in antibiotic free medium to give 90% confluency the next day. Lipofection was carried out according to manufacturers' protocol. For each well of a 12 well plate 1.6 $\mu$ g of DNA (0.8 $\mu$ g pcDNAZeoOTC and 0.8 $\mu$ g pcDNAHygArgI) was diluted into 100 $\mu$ l  $\alpha$ -MEM medium (serum and antibiotic free). 4 $\mu$ l lipofectamine 2000 reagent was also diluted in 100 $\mu$ l  $\alpha$ -MEM medium (serum and antibiotic free). After 5 minutes, the two aliquots were mixed gently and incubated at room temperature for 20 minutes. This was then added to a well containing 800 $\mu$ l  $\alpha$ -MEM medium (serum and antibiotic free). This was done for 10 wells. A pcDNA3.1LacZ containing construct was used to assess transfection efficiency, and for a negative control the empty vector pcDNA3.1 was used. The cells were incubated as for normal tissue culture and after 4 hours, the medium was removed and replaced with complete medium without antibiotics for 24 hours. Cells transfected with pcDNAZeoOTC and pcDNAHygArgI were assessed for urea cycle function, see below (section 5.3.2.3). In addition, 24 hours after the transfection, cells transfected with pcDNA3.1LacZ were used in the  $\beta$ -galactosidase assay, described below.

#### **5.3.2.2 $\beta$ -galactosidase Assay**

This assay was carried out to assess transfection efficiency using the plasmid pcDNALacZ.

##### *Materials*

Fixative: 0.05% glutaraldehyde (50% frozen stock) in 1xPBS EM grade

X-gal solution: 40mg/ml X-gal in Dimethyl formamide

X-gal base: 10mM Potassium ferricyanide, 10mM Potassium ferrocyanide, 2mM Magnesium Chloride in 1xPBS

#### *Method*

After removing the medium, 1ml of fixative was added to the cells (per well of a 6-well plate) for 15 minutes at room temperature. The cells were then washed 3x over 15 minutes with 1xPBS. In the meantime, 250µL of X-gal solution was added to 10ml of X-gal base. 1ml of this X-gal solution was added to each well and the plate sealed, with parafilm around the side, and incubated at 37°C overnight. Volumes were adjusted according to well size.

#### **5.3.2.3 Metabolic Labelling of Transient Transfectants**

HepG2 cells transfected with pcDNAZeoOTC and pcDNAHygArgI were assessed for urea cycle function and functionality of the constructs. After the 4-hour incubation with the two constructs, the HepG2 cells were incubated for 24 hours with <sup>14</sup>C-NaHCO<sub>3</sub>. Conditioned medium and cells were harvested to determine <sup>14</sup>C-urea production, and OTC and arginase I function by TLC as in Chapter 3, section 3.1.1.3, page 64.

#### **5.3.2.4 Stable Transfection of HepG2 cells with pcDNAZeoOTC and pcDNAHygArgI**

HepG2 cells were transfected with the constructs pcDNAZeoOTC and pcDNAHygArgI using Lipofectamine 2000 reagent, as described above. The cells were then placed under selective pressure using antibiotics so as to create stable dual transfectants (DT). In addition, HepG2 cells were transfected with either 1.6µg pcDNAZeoOTC or 1.6µg pcDNAHygArgI to give single transfectants which were then grown in hygromycin and zeocin respectively to determine whether there was overlap in antibiotic resistance of the antibiotic selectable markers of the constructs.

#### *Materials*

Hygromycin (50mg/ml stock) (Invitrogen)

Zeocin (100mg/ml stock) (Invitrogen)

α-MEM complete medium

18 gauge needle

Cell freezing mix: 9 parts FCS: 1 part DMSO

#### *Method*

24 hours after the transfection of the HepG2 cells with both pcDNAZeoOTC and pcDNAHygArgI, dual transfected cells were selected for by, washing each well 3x with

HBSS, adding 1ml complete medium and scraping cells with a cell scraper, syringing this 3x and placing the whole volume into a new well of a 12-well plate (passage (p) +1). The following day the medium was changed to antibiotic selection medium. Antibiotic selection medium was made by adding hygromycin at 0.04mg/ml and zeocin at 1µg/ml final concentrations to  $\alpha$ -MEM complete medium. The cells were then kept in this medium for 1 week, with medium changes every two days. At this time, cells that had not been transfected with both constructs had been killed by the antibiotics, and in order to maintain the few cells that remained, the antibiotics were added to a mixture of 50% complete medium and 50% conditioned medium from HepG2 cells (50:50 medium). The medium was changed every three days until colonies of cells started to grow. After three weeks, single colonies were picked and cells disaggregated with a needle. A cell count was taken and then half the cells placed in a 12-well plate (mixed population cells p+2). The medium was changed every 4 days with 50:50 medium until colonies grew. For clonal cells, single cell dilutions were placed in 200µl 50:50 medium in a 96-well plate. Seeding of single cells was observed using a microscope and cells maintained until colonies formed, which were then expanded and aliquots frozen.

The mixed population cells were maintained in standard antibiotic selection medium and were expanded and aliquots frozen in 50% freezing mix, 50% complete medium. In addition, mixed population single transfectants were expanded and aliquots frozen in the same way as for dual transfectants. Clonal arginase I transfected cells were maintained in hygromycin only containing medium, however, OTC cells did not survive clonal selection.

#### **5.3.2.5 OTC and Arginase I Gene Expression in Stable Dual Transfectants**

OTC and arginase I gene expression in monolayer stable dual transfectants p+20 was determined by Real Time RT-PCR as previously described in section page, with primers as in section page.

#### **5.3.2.6 Urea Cycle Expression in Stable Dual Transfectants**

*De novo* urea synthesis in monolayer cells was determined by  $^{15}\text{NH}_4\text{Cl}$  metabolic labelling and GC-MS as described in Chapter 3 section 3.1.1.2. Mixed population stable dual transfectants p+20 were also encapsulated in alginate and cultured in a RCCS in order to determine whether 3D culture with increased mass transfer of nutrients and removal of potential toxins could up-regulate urea cycle function. These encapsulated cells were then placed in a FBB, to imitate a BAL device running for 8 hours, and urea cycle function assessed by  $^{15}\text{NH}_4\text{Cl}$  metabolic labelling and GC-MS. Cell viability and albumin secretion over that time period were also assessed.

#### **5.3.2.6.1 Culture of Dual Transfectants and HepG2 Cells in a Rotary Cell Culture System**

The RCCS provides a low shear/high mass transfer environment for cells and tissue constructs. Alginate encapsulated cells were cultured in the RCCS for seven days, with medium replaced every day, prior to their transfer to the FBB for 8 hours.

##### *Materials*

Alginate encapsulated HepG2 cells and Dual Transfectants (see Chapter 2)

4 Station Rotary Cell Culture System (Synthecon)

HG Medium supplemented with Zeocin and Hygromycin as above

##### *Methods*

Cells were seeded in the alginate at  $1.0 \times 10^6$  cells/ml. Following alginate encapsulation, the beads produced were cultured in the RCCS for 7 days. Before use the vessel was filled with culture medium via the fill port and attached to the RCCS base unit in an incubator to allow equilibration of the vessel. To seed the vessel with beads, 15ml of beads were resuspended in 50ml of HG medium and added to the vessel via the fill port using a sterile Pasteur pipette. The vessel was then completely filled with medium (~500ml) and the cap replaced on the fill port. Two 20ml syringes were attached to the syringe ports, one containing culture medium. Air bubbles in the vessel can be aspirated into the empty syringe and replaced with medium from the other syringe. Filled vessels screw onto the RCCS base which is placed in a humidified incubator at 37°C and 5% CO<sub>2</sub>. The medium was changed every other day with 250ml fresh HG medium. The rotation speed of the vessels was adjusted to ensure suspension of the cultures, 7.5-8.5rpm was sufficient for alginate bead cultures

#### **5.3.2.6.2 Urea Production of Dual Transfectants and HepG2 Cells in a Fluidised Bed Bioreactor**

Miniature FBB columns were used to mimic the effects of such a system on the encapsulated cells in order to determine their detoxificatory capacity over 8 hours. This was setup by more experienced members of the group. Functionality of the cells was assessed by <sup>15</sup>NH<sub>4</sub>Cl metabolic labelling and GC-MS as previously described in Chapter 3, section 31.1.2, page 60. Dual transfectants were compared to HepG2 cells, with two columns for each cell type.

##### *Materials*

Alginate beads from RCCS

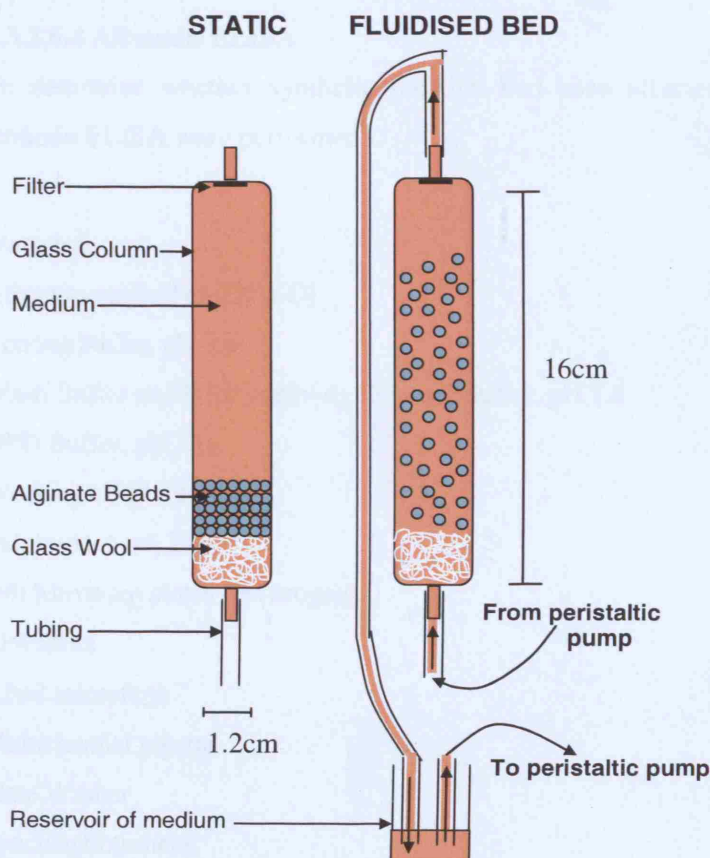
Glass columns

HG medium with 1mM <sup>15</sup>NH<sub>4</sub>Cl added

Glass wool  
 Filter  
 Peristaltic pump  
 50ml tube for reservoir  
 Parafilm  
 Incubator at 37°C and 5% CO<sub>2</sub>

### Method

Each column was blocked with glass wool at the bottom and a filter at the top to stop alginate beads escaping. 8ml of alginate beads were added to each column with 33ml of total medium, including the medium in the reservoir of each column (Figure 5.5). Parafilm was used to seal the top of the reservoir. The peristaltic pump pumped medium from the reservoir into the column, raising the bead bed, with the excess medium draining back into the reservoir. The beads were perfused with 1mM <sup>15</sup>NH<sub>4</sub>Cl spiked medium for 8 hours, after which time the medium and alginate beads were collected. Medium and bead samples were also collected prior to the start of the perfusion (time 0 hours). The beads were assessed for cell viability using the assay described below. Cells were removed from alginate and nuclei counted, as described in Chapter 2, section 2.1.2.1 page 48 and section 2.1.4, page 50, respectively. The medium collected was analysed for <sup>15</sup>N-urea using GC-MS, as previously described in Chapter 3. The medium was also analysed for albumin content, as described below (page 115).



*Figure 5.5: Diagram of the fluidised bed bioreactor. When static, the beads form a compact bed, when fluidised, the flow of medium causes the bed to rise and the beads are suspended in the medium.*

#### **5.3.2.6.3 Viability Stain**

Cell viability can be determined using the dyes fluorescein diacetate (FDA) and propidium iodide (PI) as live cells actively metabolise FDA to the fluorescent fluorescein but exclude PI, whilst dead cells cannot metabolise FDA and are porous to PI.

##### *Materials*

FDA 1mg/ml

PI 1mg/ml

PBS with calcium and magnesium

Foil

Microscope slides

##### *Method*

250µl of alginate beads were washed in 1ml PBS twice and then resuspended in 0.5ml PBS. 10µlFDA and 20µlPI were added, the sample gently agitated, and incubated in the dark for 1.5minutes. The beads were then washed twice again with PBS and the beads resuspended in 0.5ml PBS. Beads were placed on a microscope slide, excess liquid removed and microscope images taken using Lucia microscope software under phase contrast and with the corresponding fluorescent filters.

#### **5.3.2.6.4 Albumin ELISA**

To determine whether synthetic function had been affected in the stable transfectants, an albumin ELISA were performed.

##### *Materials*

Albumin antibodies (DAKO)

Coating buffer, pH 9.6

Wash Buffer and HRP Antibody Dilution Buffer, pH 7.4

OPD Buffer, pH 5.0

Working OPD solution

Sulphuric Acid, 2M

F96 Maxisorp plates (Invitrogen)

LP4 tubes

1.5ml microfuge

Multichannel pipette

Plate Washer

Spectrophotometer

### Method

The 96-well plate was coated with the first antibody, diluted to 10mg/L in coating buffer. Using a multichannel pipette 100µl was added to each well, the plate wrapped in clingfilm and placed in oven at 37°C for 2 hours. The plate was washed 3x with PBS/Tween using a plate washer. The standards for the albumin ELISA were prepared as follows: 200, 150, 100, 75, 50, 25, 12.5, 6.25 and 3.125ng/ml. To the plate, 100µl of standards and samples in triplicate were added, the plate wrapped in clingfilm, incubated at 37 °C for 1 hour then the wash repeated as above. The HRP linked antibody was diluted 1 in 8000 in PBS/Tween and 100µl was added to each well. The plate was again wrapped in clingfilm, incubated at 37°C for 1 hour then washed 5x with PBS/Tween. 100µl of OPD solution was added to each well at timed intervals, starting with the addition to the first row. The plate was covered in foil for 3-4 minutes to allow for colour development, then 50µl of acid was added, again at timed intervals to stop the reaction. The plate was read on the plate reader at 492nm. The values were corrected for cell number.

## 5.3.3 Results

### 5.3.3.1 Amplified OTC and Arginase I cDNA from Human Liver

As can be seen in Figure 5.6, OTC and arginase I cDNA were successfully amplified from human liver; this was done in duplicate.

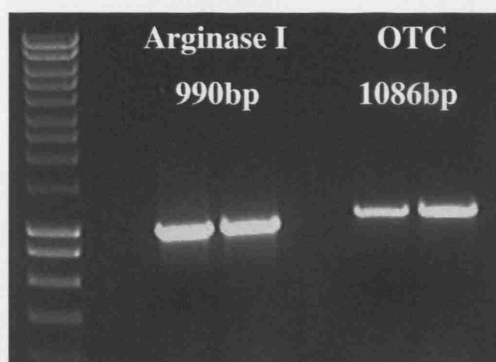


Figure 5.6: OTC and arginase I cDNA amplified from human liver.

### 5.3.3.2 Bacterial Colonies Positive for pcDNAZeoOTC and pcDNAHygArgI

The colonies obtained for pcDNAZeoOTC and pcDNAHygArgI were analysed by PCR and the products run on a gel. Since the coding region of OTC and arginase I are 1065bp and 969bp, respectively, the addition of the MCS primers will give PCR products of 1118bp for OTC and 1022bp for arginase I (Figure 5.7, page 117).



### 5.3.3.3 Sequencing of pcDNAZeoOTC and pcDNAHygArgI

A selection of the above colonies were sent for sequencing; two pcDNAZeoOTC constructs were identical to the sequence in GenBank, and one pcDNAHygArgI construct was identical to the GenBank sequence.

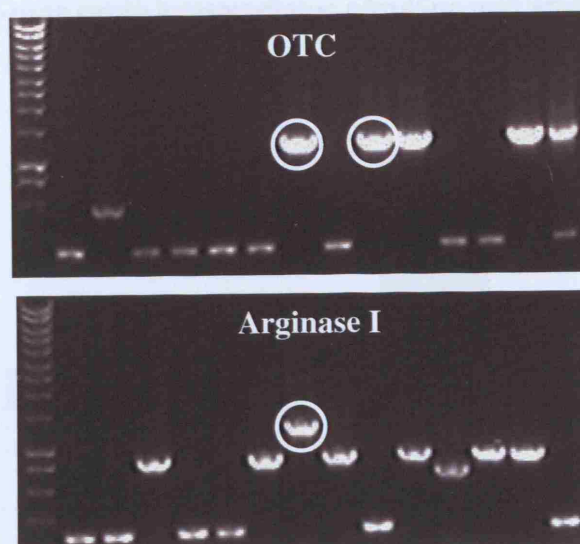


Figure 5.7: Colonies analysed by PCR for OTC and arginase I containing constructs. Circled are the bands with the correct sized PCR product for OTC arginase I.

### 5.3.3.4 Transfection Efficiency in HepG2 cells

The transfection efficiency, as assessed by transfecting HepG2 cells with the pcDNALacZ plasmid, was around 40-50% (Figure 5.8).

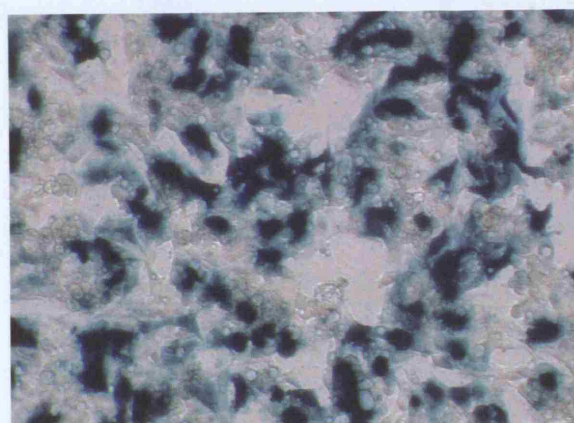


Figure 5.8: Transfection efficiency using pcDNA3.1LacZ and the  $\beta$ -galactosidase assay on HepG2 cells.

### 5.3.3.5 Urea Cycle Function in Transient Dual Transfected HepG2 cells

HepG2 cells were assessed for urea cycle function 24 hours after transfection with pcDNAZeoOTC and pcDNAHygArgI using  $^{14}\text{C}$ - $\text{NaHCO}_3$  metabolic labelling. As can be seen in Figure 5.9 (page 118), the dual transfectants are capable of making  $^{14}\text{C}$ -



arginine and to a lesser extent  $^{14}\text{C}$ -citrulline. Figure 5.10, shows that these cells are also capable of making  $^{14}\text{C}$ -urea, showing that the constructs are functional and that they can restore OTC and arginase I activity, resulting in urea cycle function.

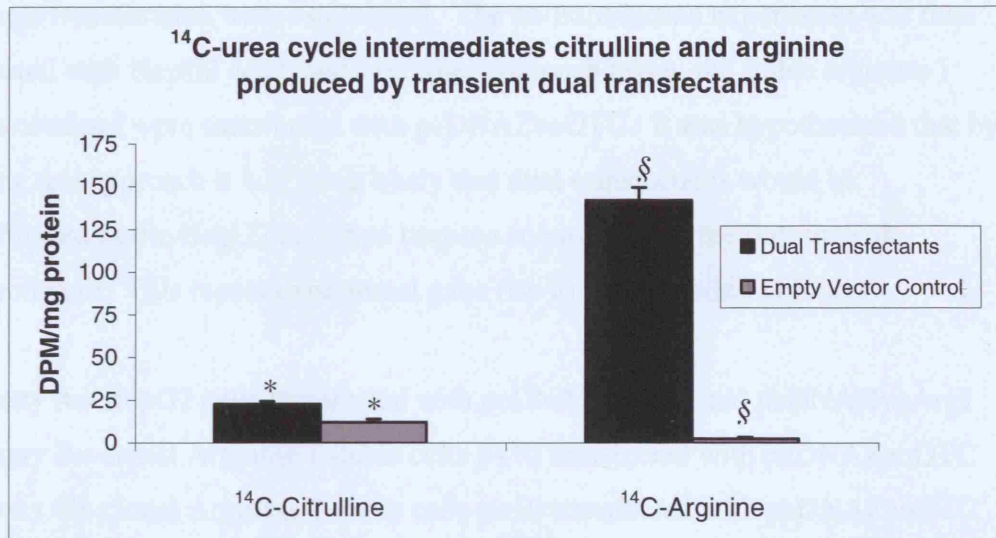


Figure 5.9:  $^{14}\text{C}$ -urea cycle intermediates produced by transient dual transfectants.  $^{14}\text{C}$ -arginine and  $^{14}\text{C}$ -citrulline are produced by transiently transfected HepG2 cells (black bar). The empty vector control counts reflect background (grey bar), ( $n=3$ , mean $\pm$ SD, repeated twice, \* $p<0.05$ , § $p<0.005$ ).

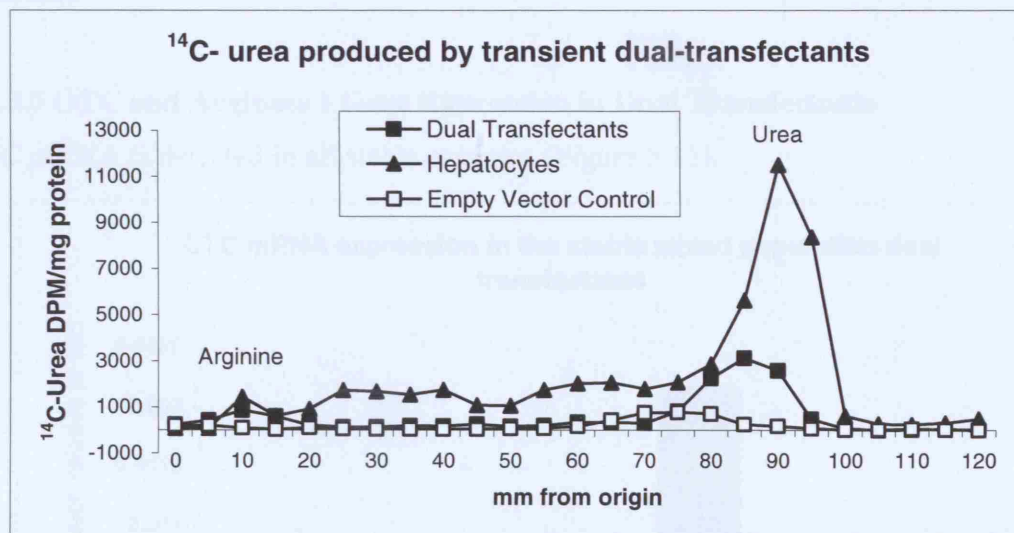


Figure 5.10:  $^{14}\text{C}$ -urea production by transient dual transfectants. A peak for  $^{14}\text{C}$ -urea is detected in transient dual transfectant conditioned medium (solid square), background counts are seen in the empty vector control (open square; a peak of urea is also seen in the hepatocyte positive control (solid triangle), ( $n=2$ , repeated twice).

### 5.3.3.6 Urea Cycle function in Stable Mixed Population Dual Transfected HepG2

#### Cells

When this experiment was first conducted, no dual transfectant colonies were isolated after the first addition of antibiotic selection medium. However, arginase I single transfectants were established. The co-transfection experiment was then repeated with HepG2 cells, and one other approach taken; the stable arginase I cells obtained were transfected with pcDNAZeoOTC. It was hypothesized that by taking this approach it was more likely that dual transfectants would be established as the HepG2 cells had become accustomed to the presence of hygromycin. This repeat experiment gave rise to the following colonies:

**Colony A**= HepG2 cells transfected with pcDNAZeoOTC and pcDNAHygArgI

**Colony B**= clonal Arginase I stable cells p+10 transfected with pcDNAZeoOTC

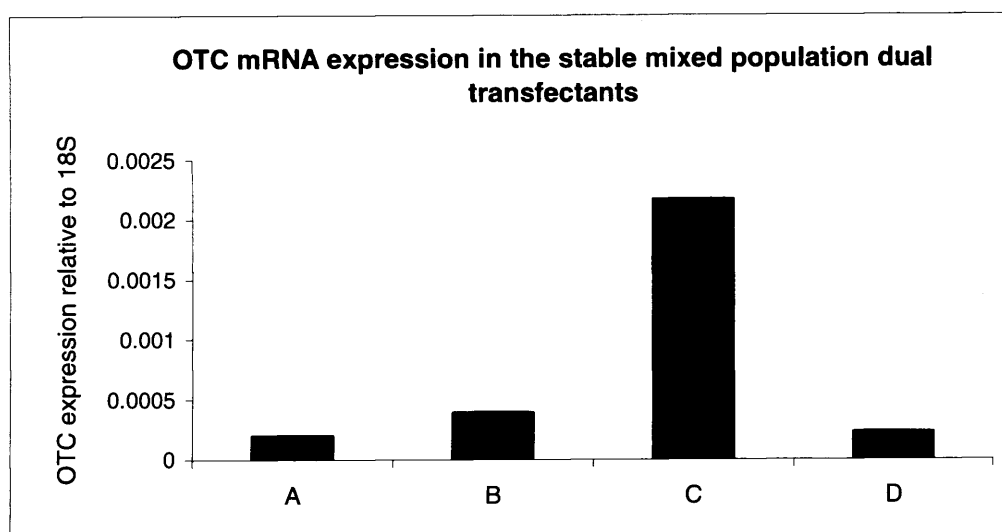
**Colony C**= clonal Arginase I stable cells p+10 transfected with pcDNAZeoOTC

**Colony D**= clonal Arginase I stable cells p+10 transfected with pcDNAZeoOTC

Single transfectants could not grow in the wrong antibiotic, i.e. HepG2 cells transfected with pcDNAZeoOTC could not grow when cultured in hygromycin, and HepG2 cells transfected with pcDNAZeoArgI could not grow when cultured in zeocin.

### 5.3.3.7 OTC and Arginase I Gene Expression in Dual Transfectants

OTC mRNA is detected in all stable colonies (Figure 5.11).



*Figure 5.11: OTC mRNA expression in stable mixed population dual transfectants A, B, C, and D (n=2).*

Interestingly however, arginase I mRNA expression is only present in colony A, and is not found in colonies B,C, and D (Figure 5.12).



Figure 5.12: Arginase I mRNA expression in stable mixed population dual transfectants, (n=2).

#### 5.3.3.8 Urea Production by Dual Transfectants

GC-MS for urea production showed that there was some  $^{15}\text{N}$ -urea produced from  $^{15}\text{NH}_4\text{Cl}$  metabolic labelling by all four cell lines (Figure 5.13).

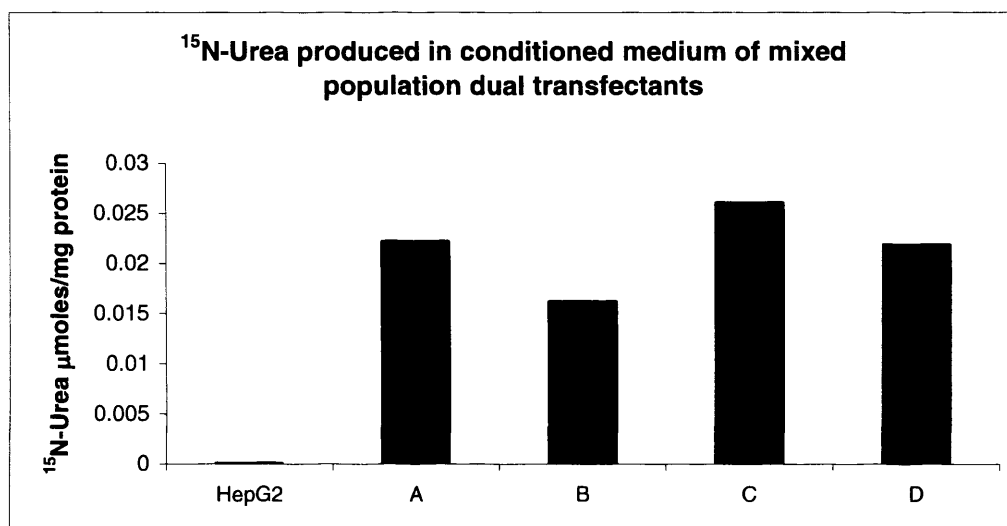
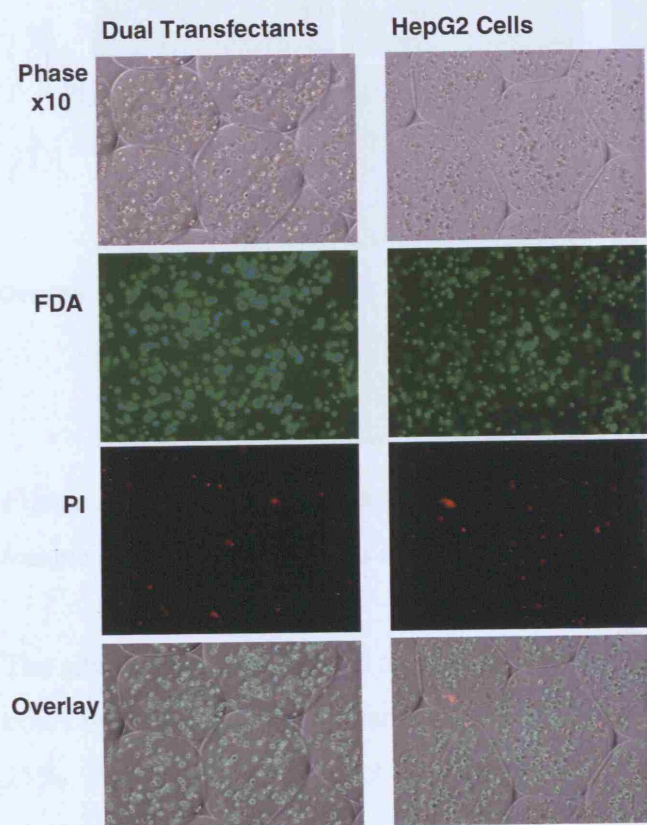


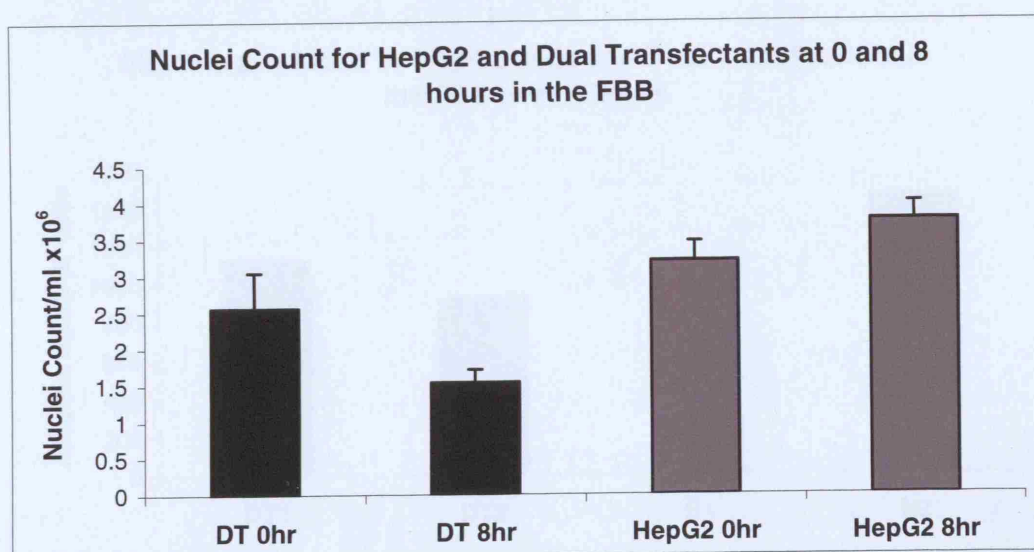
Figure 5.13:  $^{15}\text{N}$ -labelled urea production by monolayer dual transfectants. Urea could be detected in conditioned medium of the dual transfectants, albeit very small amounts, (n=3, mean $\pm$ SD). No urea is detected in HepG2 cell medium.

### 5.3.3.8 Performance of Dual Transfectants (Colony A) and HepG2 Cells in a FBB

Colony A was chosen for the FBB experiments as it expressed both OTC and arginase I. Figure 5.14 shows the viability of the cells just prior to placing in RCCS for 7 days. The nuclei count (Figure 5.15) and the cell viability stain (Figure 5.16, page 122), show the survival of HepG2 cells and dual transfectants over the 8 hour period in the FBB. HepG2 cells maintained viability over the 8 hours in the FBB whereas cell viability dropped slightly in the dual transfectants.



*Figure 5.14: Cell viability staining of dual transfectants (DT) and HepG2 cells in RCCS. Photos were taken just after encapsulation and prior to adding beads to RCCS, x10 magnification.*



*Figure 5.15: Nuclei count of dual transfectants (DT) and HepG2 cells in the FBB. Counts were taken at the start (0hr) and end (8hr) of the experiment ( $n=5$ , mean $\pm$ SD).*



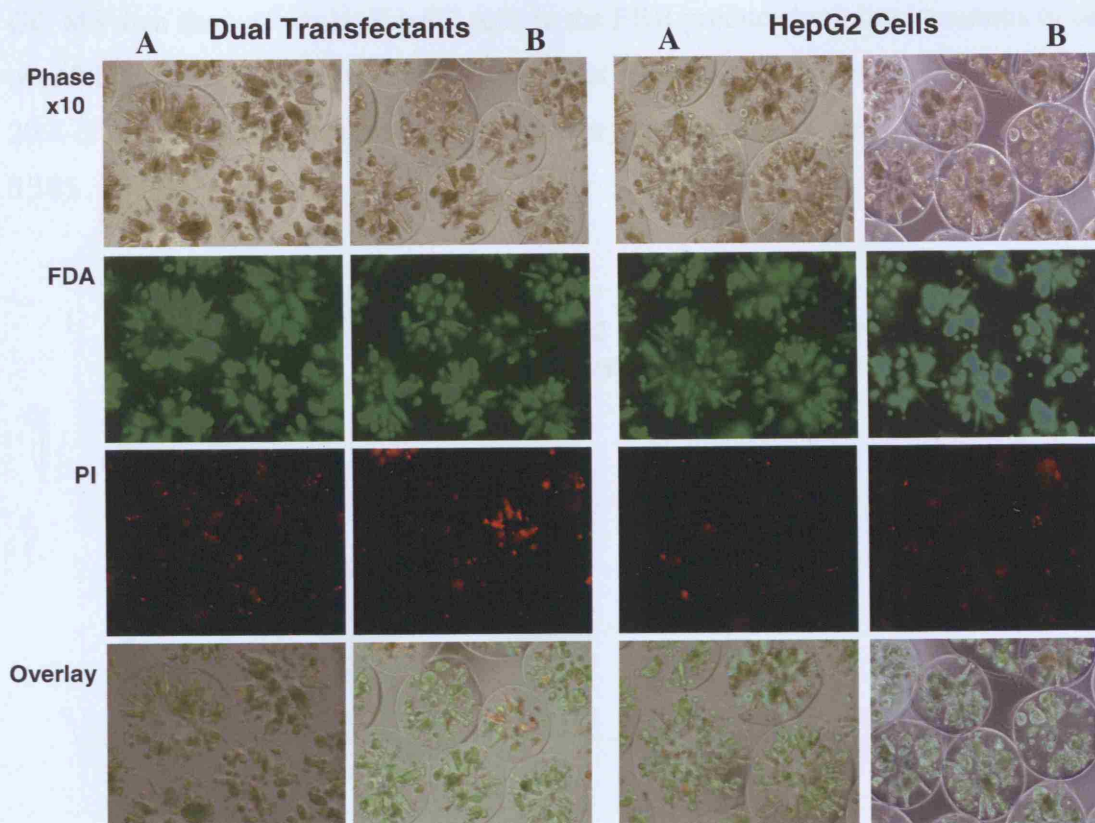


Figure 5.16: Cell viability staining of dual transfectants and HepG2 cells in FBB. Images were taken at 0 hours (column A) and 8 hours (column B), x10 magnification.

The albumin ELISA showed that more albumin was secreted by HepG2 cells over 8 hours than the dual transfectants, although on average the decrease was no more than 25%. Figure 5.17 shows each of the columns, with column H1 producing very low amounts.

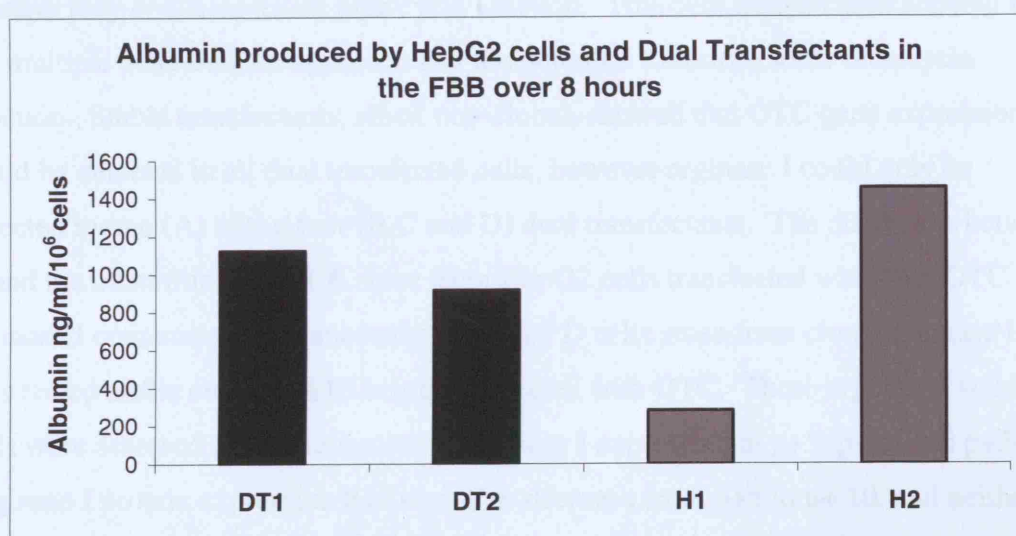


Figure 5.17: Albumin production by dual transfectants (DT) and HepG2 cells (H) in the FBB over 8 hours. Results from duplicate columns (1 and 2) are shown.

GC-MS data showed whilst HepG2 cells in the FBB produced minimal amounts of urea over 8 hours, the dual transfectants produced at least 10-fold the amount, and about 20% of the amount of urea produced by primary human hepatocytes in culture (Figure 5.18).

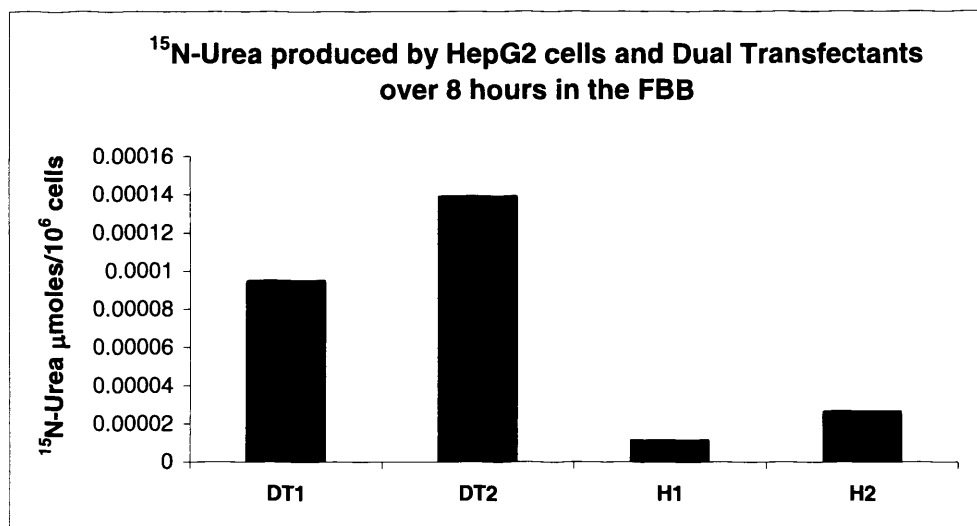


Figure 5.18: <sup>15</sup>N-urea production by dual transfectants (DT) and HepG2 cells (H) in the FBB. Counts were taken at the beginning (0 hours) and end (8 hours) of the experiment.

#### 5.4.4 Discussion

Once it was established that both pcDNAZeoOTC and pcDNAHygArgI constructs had been isolated with correct sequences, they could be assessed for functional activity and whether they could replenish urea cycle function. Transient transfectants showed that the multiple gene transfer approach had the potential to restore some urea cycle function. Stable transfectants, albeit non-clonal, showed that OTC gene expression could be detected in all dual transfected cells, however arginase I could only be detected in one (A) of the four (B,C and D) dual transfectants. The difference between A and the other three is that A arose from HepG2 cells transfected with both OTC and arginase I constructs simultaneously; B, C and D cells arose from clonal arginase I transfected stable cells at p+10 being transfected with OTC. These arginase I stable cells were assessed for the retention of arginase I expression at p+5, p+10 and p+20. Arginase I protein expression had started to decrease from p+5 to p+ 10, and neither protein nor mRNA was found in p+20 cells, even though cells were constantly maintained under antibiotic selection. Despite this, all four cell lines, A-D, when labelled with <sup>15</sup>NH<sub>4</sub>Cl, produced <sup>15</sup>N-urea. In the case of A, which expresses both OTC

and arginase I, it is likely that urea cycle function has been restored. In the case of B-D, one possible explanation could be that since these cells express OTC only, they are capable of incorporating labelled ammonia into the urea cycle only as far as arginine. The  $^{15}\text{N}$ -labelled arginine can then be used by arginase II to produce  $^{15}\text{N}$ -labelled urea.

Data from the FBB experiment using the dual transfectants (colony A) showed promising results. Albumin synthesis was lower, although not greatly so. Labelled urea production was increased by the 3D culture system over monolayer culture. The difficulties in using the small scale setup of a FBB may have affected cell viability; it was difficult to control the oxygenation of the medium and at times it would become slightly too acidic or alkali. In addition, at the time of the experiment, the cells had already reached p+20 and had probably already lost some function. Despite these problems however, the results are encouraging in that urea was produced by these cells.

## **5.5 Conclusion**

Various ways in which the urea cycle could be reinstated were investigated. Ammonia detoxification via the urea cycle may be necessary in order to maintain a physiological response to increased ammonia, as opposed to increasing the production of glutamine from ammonia via glutamine synthase which may have a compounding effect on hepatic encephalopathy,

Demethylation was considered, and resulted in the up-regulation of OTC expression. However, it is not feasible to culture cells in a potent carcinogen prior to their use in a BAL device, and it is unlikely that once the demethylating agent is removed OTC expression will be maintained.

The next approach was to culture cells in low arginine, which according to some reports could restore OTC expression and urea cycle function. A small increase in OTC expression was observed, however, HepG2 cells did not adapt well to low arginine and after some time stopped growing.

The final approach involved restoring the expression of OTC and arginase I by multiple gene transfer. This gave some promising results in that cells transfected with either both of these genes or OTC alone had the ability to convert labelled ammonia into

labelled urea. Data from cells maintained in culture for over 20 passages did show however, that expression of the transfected genes were lost over time. Further studies need to be carried out to optimize these procedures. These are discussed in Chapter 6.



## **Chapter 6: Conclusion**

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This chapter summarizes the main findings of chapters 3, 4 and 5 and further explores the possible reasons and implications of the findings. Lastly, future work and considerations are discussed.

### **6.1 Characterizing and Restoring Urea Cycle Function in HepG2 and C3A Cells**

It is known that HepG2 cells do not possess a functional urea cycle as they do not produce urea. C3A cells, a derivative of HepG2 cells, have been shown to produce urea. However, the reasons behind the lack of function in HepG2 cells had not been elucidated. In addition, C3A cells could offer a suitable alternative as regards ammonia detoxification for our BAL device.

#### **6.1.1 Summary of Main Findings**

The findings of chapter 3 conclusively showed by stable isotope labelling that urea cannot be produced from ammonia by HepG2 and C3A cells, and that the urea cycle is not functional due to the absence of two genes, OTC and arginase I, as revealed by both mRNA and enzyme assays; three other urea cycle genes, CPSI, AS and AL, were expressed to some degree in the two cell types. It is interesting that unlabelled urea was produced by C3A cells however, although it was clear that it could not arise from urea cycle function and investigations were continued in chapter 4. Results showed that urea was produced via arginase II, an enzyme involved in, amongst other things, polyamine synthesis. Importantly arginase II was expressed at greater levels in C3A cells than in HepG2 cells, and this probably accounts for the difference in urea production by these two cell lines. Since C3A cells did not offer a suitable replacement for HepG2 cells, investigations into whether urea cycle function could be restored in HepG2 cells, the cell of choice currently used in the development of our BAL device, were carried out. Chapter 5 showed that there was some limited success, in this regard, with attempts to adapt HepG2 cells to arginine deficient medium and by altering gene methylation patterns. The most promising approach however, was offered by gene transfer; cells both transiently and stably transfected with OTC and arginase I, resulted in complete urea cycle function, as shown by stable metabolic labelling studies.

Some interesting observations and questions were raised by our findings, and these are posed and answered below.

### **6.1.2 Why are OTC and arginase I down-regulated but CPSI, AS and AL still expressed?**

Considering the data as a whole, it may be possible that being hepatoma derived, HepG2 and C3A cells have no use for the urea cycle; an energy expensive pathway that utilizes possible useful compounds to produce urea, an unusable product. By removing OTC and arginase I, the pathway is prevented.

One reason why OTC is targeted for down-regulation could be that a lack of OTC activity would prevent the incorporation of ornithine into citrulline, and could allow for ornithine to be available for polyamine biosynthesis via ornithine decarboxylase. The role of polyamines is essential to cell proliferation and cell cycle progression (146;147). Moreover, preventing OTC and arginase I activity, allows for a potential increase in carbamoyl phosphate via CPSI, together with a reduced requirement for aspartate for the conversion of citrulline to argininosuccinate, and this would allow for increased pyrimidine biosynthesis from carbamoyl phosphate and aspartate (75;92). A lack of arginase I may also allow for arginine to be diverted to NO synthesis and/or agmatine synthesis for the further production of polyamines via arginine decarboxylase (99;148;149).

The presence of the three remaining enzymes, CPSI, AS and AL, albeit at levels lower than in primary human hepatocytes or human liver, may not be because they are left over from the now redundant urea cycle, but because these three genes have alternative roles to play in the cell. It may be expected therefore, that the level at which these genes and proteins are expressed are applicable to these alternate functions. This is likely to be the case with AS and AL, which exist as part of cycle that generates nitric oxide. The levels at which these two genes need to be expressed for the citrulline/NO cycle is far less than the levels required when expressed for the purposes of the urea cycle. It has been observed that whilst AS and AL are normally highly expressed in cells which have urea cycle function, cells that do not, express these two genes at very low levels for the purposes of nitric oxide synthesis (91).

CPSI may be required for generating carbamoyl phosphate for pyrimidine biosynthesis

as it may have functional overlap with CPSII, which exists solely for this purpose (75). The difference between the two enzymes is that whilst CPSI utilises ammonium and bicarbonate ions to generate carbamoyl phosphate, CPSII utilises glutamine and bicarbonate ions (77;150;151), Figure 6.1, page 131. Therefore, by retaining CPSI, HepG2 and C3A cells may utilise this enzyme to control ammonia and carbamoyl phosphate levels, as has been previously shown (77;92).

### **6.1.3 What is happening to the ammonia?**

Whilst HepG2 cells have been shown to remain unaffected by high ammonia levels, the question of how much ammonia these cells make and how much they use remain. They do, however, seem to use ammonia to their advantage through GS activity (and possibly through CPSI as previously mentioned), an enzyme usually reserved for perivenous hepatocytes, which has a high affinity for ammonia and where it is converted into glutamine. This may be a preferred pathway of ammonia detoxification for both HepG2 and C3A cells as glutamine would be required for cellular proliferation (48;118).

As HepG2 and C3A cells are hepatoma derived, an increase in the synthesis of glutamine may be a feature of higher rates of proliferation. It may be expected that other genes involved in proliferation, such as those required for increased polyamine, pyrimidine and purine production may also be upregulated. These genes include: carbamoyl phosphate synthase II (CPSII), ornithine decarboxylase (ODC), and glutaminase, in addition to the previously investigated genes: arginase II, ADC and agmatinase. In addition to the data presented in previous chapters, Real Time RT-PCR analysis of these additional genes was carried out in order to assess the likelihood of the up-regulation of metabolic pathways required for cell proliferation.

A summary of the function of each of these genes and the results obtained are listed below. The figures are presented in Appendix I.

- i) GS: this gene is normally expressed in perivenous hepatocytes where it converts ammonia that has escaped from periportal hepatocytes into glutamine that re-enters the circulation. It has been shown to be expressed in HepG2 cells and other cells with high rates of proliferation (48). Our results showed that GS expression in HepG2 cells was higher than in

hepatocytes or human liver, and expression was even greater in 3D-culture. GS expression in C3A cells was similar to that in hepatocytes and the liver, Figure I.1, page 134. Interestingly, hepatocytes with added growth factors displayed GS expression, whereas those cultured without growth factors did not.

- ii) Glutaminase: two glutaminase isoforms exist, kidney-type and liver-type. Located in the mitochondria, they are responsible for converting glutamine to glutamate and ammonia, to generate ATP. It is thought that kidney-type glutaminase, which has a higher affinity for glutamine than the liver-type, is prevalent in hepatoma cells due to their higher growth rates, and as a consequence liver-type glutaminase is down-regulated. Interestingly, it is thought that expression of liver-type glutaminase may be linked to urea cycle gene expression (152). Real Time RT-PCR results showed that kidney-type glutaminase mRNA expression was elevated in HepG2 and C3A monolayer cells in comparison to hepatocytes and liver, and this increase was amplified by 3D culture, Figure I.2, page 135.
- iii) CPSII: an isoform of CPSI, CPSII is expressed in the cytosol for the purposes of pyrimidine biosynthesis from the conversion of glutamine and bicarbonate ions, where it forms a trimer with aspartate transcarbamylase and dihydroorotase (153;154). Expression data (mRNA) showed that CPSII was elevated in HepG2 cells in comparison to liver. C3A cells also showed a small increase in monolayer culture, with greater increase observed in cells in 3D culture. Again it was interesting to note that hepatocytes with growth factors had far higher levels of CPSII mRNA than those without, Figure I.3, page 135. This has previously been seen in rat hepatoma cells with higher rates cell growth and proliferation (155-157).
- iv) ODC: expressed in the cytosol, it utilizes the ornithine produced by arginase II, that is exported from the mitochondria, for polyamine synthesis, starting with putrescine (158). ODC mRNA was expressed at higher levels in monolayer HepG2 and C3A cells in comparison to hepatocytes and liver, but these levels were reduced when cells were grown in 3D culture (Figure I.4, page 136).

In addition, to highlight results previously obtained in Chapter 4 (page 92) for:

1. Arginase II, a mitochondrial enzyme that converts arginine to ornithine for polyamine glutamate and proline synthesis, which may be responsible for regulating the availability of arginine for NO synthesis (105).
2. Agmatinase (responsible for the conversion of agmatine to putrescine, a polyamine (100-102).

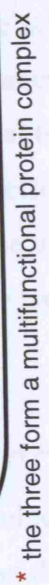
Both genes were expressed at higher levels in HepG2 and C3A cells than in primary human hepatocytes or human liver. Arginase II levels in hepatocytes cultured with growth factors were higher than cells cultured in the absence of these factors.

Our findings may be supportive of the hypothesis that the substrates glutamine, arginine, ornithine and carbamoyl phosphate are preferentially used for the enhancement of cell proliferation, which is expected of a tumour cell line.

Interestingly, although the data sets are not complete, a similar trend was seen with primary human hepatocytes cultured with the HGF and EGF, indicating that growth stimuli cause a similar effect on non-transformed cells (159).

The metabolic pathways discussed in this thesis are brought together in Figure 6.1 (page 131), which covers ammonia detoxification, glutamine metabolism, the NO cycle and purine, pyrimidine and polyamine synthesis.

## Cytoplasm



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## 6.2 Future Work and Considerations

Chapter 5 showed that the most promising avenue to the restoration of ammonia detoxification via urea cycle function was the gene transfer of OTC and arginase I into HepG2 cells. Whilst this approach was successful to some degree in transiently transfected cells and low passage number stable transfectants, as passage number increased the expression of OTC and arginase I diminished. This is not uncommon, as mammalian cells can down-regulate non-mammalian promoters, in this case CMV, by methylation. It may be possible to test whether this is the case by exposing cells with diminished transfected gene expression to a demethylating agent such as 5-azacytidine to observe whether some expression can be regained. However, for the long term culture of cells, and for cells to be used in a bioartificial liver device, this is not a suitable approach, due to: the toxicity of 5-azacytidine, the impracticalities of carrying this out on a large scale, the detrimental effects of 5-azacytidine to the cells over prolonged exposure, and because of the likelihood that the genes will be re-silenced when the demethylating agent is removed.

Other approaches can be taken, mammalian promoters can be used, for example an albumin promoter would be very effective in the HepG2 cell line due to naturally high albumin expression; although, these too may be silenced over time. Integrative vectors may also give more consistent results than episomal plasmids. This could be achieved by linearizing plasmid vectors prior to transfection, or by using lentiviral or adeno-associated viral vectors. Integrative vectors need to be assessed to ensure that they do not insert themselves into a part of the genome that could cause detrimental effects to the cell, or cause the overexpression of undesired genes. In the context of the use of such transformed cells in BAL devices, a DNA binding membrane would be part of the device to ensure no transfer of such material to the patient.

One other possible avenue of investigation could be to transfect cells with OTC alone, rather than by using OTC and arginase I. This may probably be less stressful to the cell as it would require the use of only one selection antibiotic. This would mean that rather than producing urea from ammonia, cells could produce arginine from ammonia, and since arginine is a biosynthetic amino acid, it may be incorporated into proteins and converted to ornithine and urea by arginase II, the latter was seen when cells expressing OTC mRNA alone produced  $^{15}\text{N}$ -urea from labelling experiments. This may have a beneficial effect on the patient receiving treatment with a BAL device containing these

cells since the ammonia taken up from their blood can be turned into an amino acid the cells can use, rather than excreting urea, which could be an additional burden to patients with compromised organ function.

Lastly, essential to this work would also be the development of a sensitive and accurate assay to measure ammonia uptake. This is likely to be a GC-MS based approach, for which protocols exist in the literature. This would be necessary since the data presented show that HepG2 cells express both GS and glutaminase, and the balance between ammonia utilisation and production is not known. In addition, the contribution to the uptake of ammonia by alternative pathways needs to be determined.



## Appendix I

Real Time RT-PCR analysis of genes involved in alternative mechanisms of ammonia detoxification and cell proliferation was carried out. The genes investigated were: GS, glutaminase, CPSII and ODC.

### Methods

Real Time RT-PCR was carried out on the genes listed in Table I.1.

Table I.1: Primer sequences for GS, Glutaminase, CPSII and ODC.

Sequence	Primer forward 5'	Primer reverse 5'	Fragment bp
GS	CACCTGTAACGGATAATGGACAT	CACACACAGTAATATGGACCCTG	150
Glutaminase	GTTGGCAACAGCGAGGGCA	CTGGGATCAGACGTTTCGCAA	181
CPSII	ACTGCCGGGGGAAGTGGTGTTC	CCGAACATCATCCATTTTCATCTGGG	145
ODC	GGTAATGAAGAGTTTGACTGCC	GGCATCCTTATCATCAGAAG	104

### Results

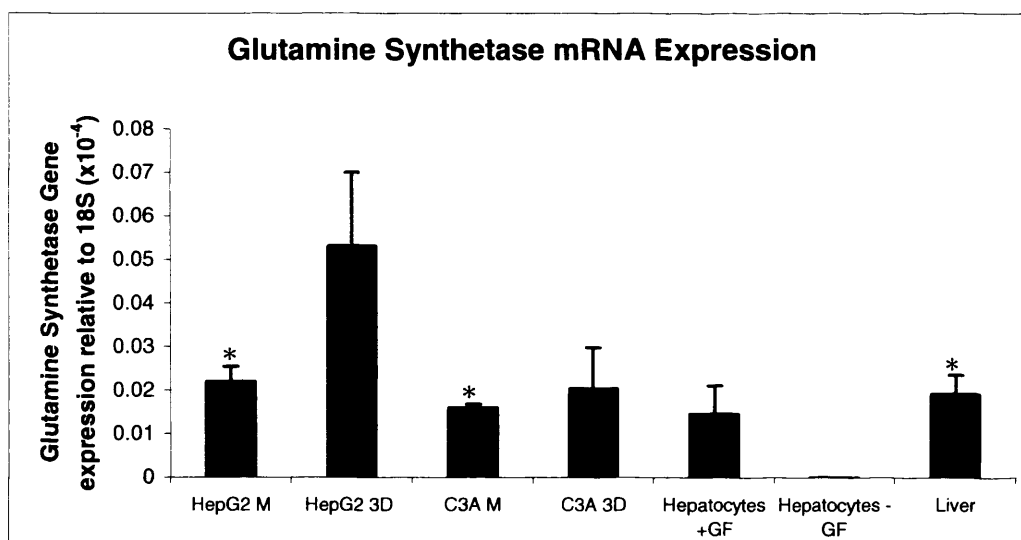


Figure I.1: Real Time RT-PCR for GS mRNA levels in HepG2 and C3A cells in comparison to primary human hepatocytes with and without growth factors (GF), and whole human liver ( $n=6$ , mean $\pm$ SD, repeated 3 times, \* $p<0.05$ ).

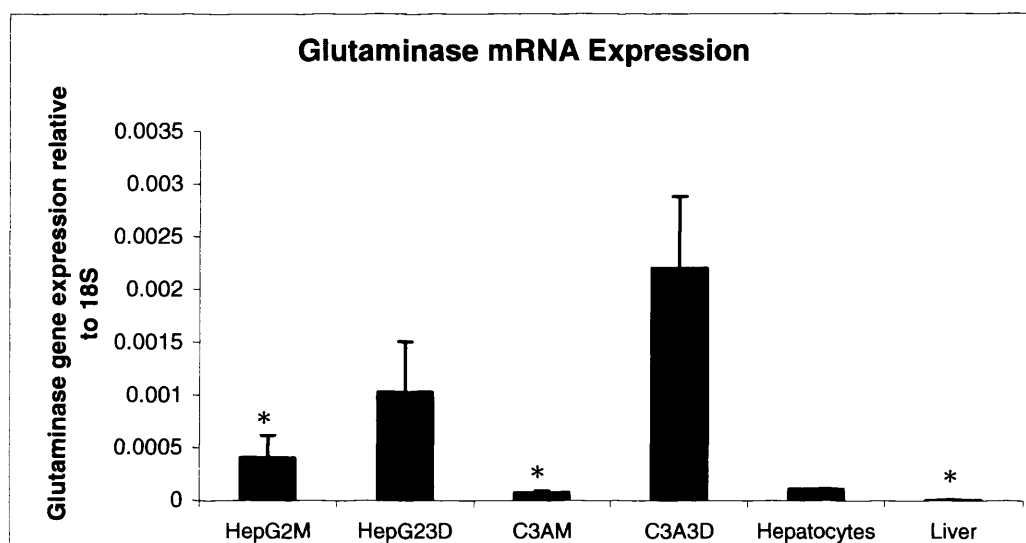


Figure I.2: Real Time RT-PCR for glutaminase mRNA in HepG2 and C3A cells where it is greater than in hepatocytes and whole human liver; expression is further enhanced by 3D culture ( $n=6$ , mean $\pm$ SD, repeated 3 times,  $*p<0.005$ ).

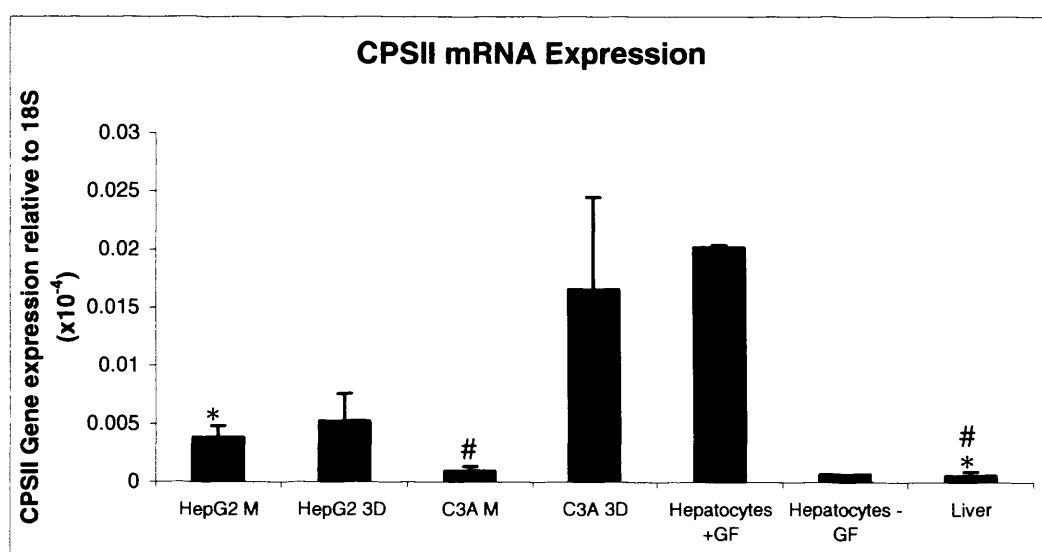
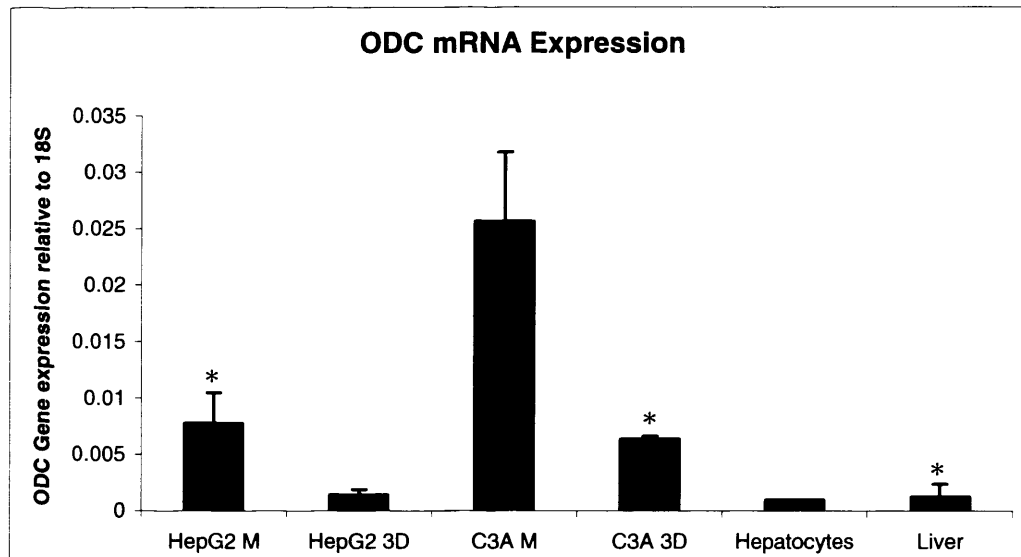


Figure I.3: Real Time RT-PCR for CPSII mRNA in HepG2 cells and C3A cells where expression is higher than in hepatocytes and human liver; levels increase further in 3D culture ( $n=6$ , mean $\pm$ SD, repeated 3 times,  $*p<0.05$ ,  $\#p=ns$ ). Culturing hepatocytes with growth factors (GF) causes a large increase in CPSII expression.



*Figure I.4: Real Time RT-PCR for ODC mRNA in HepG2 and cells is higher than in hepatocytes and human liver, although levels drop in 3D culture (n=6, mean $\pm$ SD, repeated 3 times, \* $p$ <0.05).*

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## **Publications**

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1. Ornithine transcarbamylase and arginase I deficiency are responsible for diminished urea cycle function in the human hepatoblastoma cell line HepG2.  
Mavri-Damelin D, Eaton S, Damelin LH, Rees M, Hodgson HJ, Selden C.  
Int J Biochem Cell Biol. 2007;39(3):555-64. Epub 2006 Oct 21.
  
2. Cells for bioartificial liver devices: the human hepatoma-derived cell line C3A produces urea but does not detoxify ammonia.  
Mavri-Damelin D, Damelin LH, Eaton S, Rees M, Selden C, Hodgson HJ.  
Biotech. Bioeng. 2007. Epub 2007 Aug 6.